Philipps Universität Marburg

Disentangling the Role of SHANK1 in a Mouse Model for Autism Spectrum Disorder: From Brain to Behavior

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

dem Fachbereich Psychologie der Philipps-Universität Marburg

vorgelegt von

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Marburg, 2017

Vom Fachbereich Psychologie

der Philipps-Universität Marburg als Dissertation am 27.09.2017 angenommen.

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Tag der mündlichen Prüfung: 27.09.2017

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SUMMARY

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorders characterized by persistent deficits in social communication and interaction across multiple contexts, and restricted, repetitive patterns of behavior; frequently comorbid with intellectual disability (ID). Several studies highlight immense contribution of genetic factors to disease etiology. Particularly, the SHANK family of postsynaptic proteins has emerged as promising candidates, considering that mutations in *SHANK1*, *SHANK2*, and *SHANK3* genes have repeatedly been reported in individuals with ASD. Animal models provide excellent translational tools to discover disease pathogenesis underlying behavioral and neurobiological abnormalities. This dissertation aimed at understanding these mechanisms by using the *Shank1* knockout mouse model for ASD, with an in-depth and longitudinal focus on each diagnostic symptom. Specifically, ASD-like phenotypes were investigated throughout development and across different social contexts. While social behavior was only moderately affected in mice lacking SHANK1 (Study I), evidence for communication deficits and repetitive behavior throughout development and/or across different social contexts were demonstrated in these animals (Study II&III). In conjunction with ASD – ID comorbidity, deletion of *Shank1* resulted in severe cognitive impairments (Study I). Highlighting the pivotal role of the hippocampus in this mechanism, elevated levels of learning-associated brain-derived neurotrophic factor were found in the hippocampi of *Shank1* mutants. This increase in protein expression was paralleled by alterations in epigenetic regulation (Study I). Overall, results of the studies presented here indicate that *SHANK1* is involved in ASD-relevant deficits across species. These findings further extend the knowledge on social communication and interaction, repetitive behaviors, and cognitive phenotypes displayed by the *Shank1* mouse model for ASD in an age- and sex-dependent manner, underscoring the importance of social context in ASD research.

ZUSAMMENFASSUNG *

Autismus-Spektrum- Störungen (ASS) gehören zu einer Gruppe von Entwicklungsstörungen des Nervensystems. Diese Störungen zeichnen sich durch anhaltende Abweichungen der sozialen Kommunikation und Interaktion in verschiedenen Kontexten und durch eingeschränkte, repetitive Verhaltensmustern aus. Häufig tritt eine Komorbidität mit mentalen Retardierungen auf. Viele Studien konnten eine genetische Ursache für die Entstehung dieser Krankheit verantwortlich machen. Besonders Mutationen einer Familie postsynaptischer Proteine, den SHANKs, haben sich dabei als wahrscheinliche Grundlage dieser genetischen Ursache herausgestellt, da verschiedene *SHANK*-Mutationen gehäuft in ASS-Patienten auftreten. Tiermodelle bieten eine effektive Möglichkeit, die genetischen Ursachen, die der Entstehung dieser Krankheit zugrunde liegen mit Verhaltensauffälligkeiten zu korrelieren. In dieser Dissertation wurde die Rolle von SHANK1 in der Entstehung von ASS-assoziierten Symptomen in unterschiedlichen sozialen Kontexten während der Entwicklung in einem *Shank1* knockout Mausmodell untersucht. Während ein *Shank1* knockout auf das murine Sozialverhalten nur einen mäßigen Einfluss hat (Studie I), entwickelten diese Mutanten in verschiedenen sozialen Kontexten Kommunikationsdefizite und repetitives Verhalten (Studie II&III). Darüber hinaus deutet Studie I darauf hin, dass SHANK1 eine Rolle bei der Komorbidität von ASS und der mentalen Retardierung spielt, da ein *Shank1* Knockout zu kognitiven Beeinträchtigungen führte. Diese kognitive Beeinträchtigung korrelierte mit einer erhöhten Expression von mit Lernen assoziiertem BDNF im Hippocampus, was die herausragende Rolle des Hippocampus in diesem Zusammenhang unterstreicht. Die erhöhte BDNF Expression ging weiterhin mit epigenetischen Veränderungen einher (Studie I). Zusammengefasst deuten diese Resultate auf eine Beteiligung einer *Shank1* Mutation bei der Entstehung ASS-assoziierter Defizite in der Maus hin. Diese Ergebnisse tragen zum Verständnis der Rolle von SHANK1 bei der alters- und geschlechtsabhängigen Entstehung von Abweichungen der sozialen Kommunikation und Interaktion, repetitivem Verhalten, und kognitiven Defiziten bei und unterstreichen dadurch die Bedeutung des sozialen Kontexts bei der Erforschung von ASS.

* My sincerest gratitude goes to Philipp Gobrecht for his extensive help in this translation.

1 INTRODUCTION

1.1 Autism Spectrum Disorder

In 1911, the Swiss psychiatrist Eugen Bleuler was first to introduce the term "autism" (from the Greek word *autós*, meaning "self"), to describe one of the symptoms of schizophrenia, saying:

"The most severe schizophrenics […] live in a world of their own. […] they limit contact with the outside world as much as possible. This detachment from reality with the relative and absolute predominance of the inner life, we term autism." (Bleuler, 1911)[†]

Bleuler's introduction to the concept of autism was later redefined by two pioneers; child psychiatrist Leo Kanner (1943), and pediatrician Hans Asperger (1944), which opened the doors to the research of autism today. In his very first report on several children "*whose condition differs so markedly and uniquely from anything reported so far"*, Kanner observed, albeit with certain individual variations, a number of common characteristics shared by these children who had been prospectively examined by him. Among these characteristics, he defined some as *inability to relate themselves*, *extreme autistic aloneness*, some acquiring *ability to speak at a later age*, with *monotonously repetitious behavior* and *anxiously obsessive desire for the maintenance of sameness* (Kanner, 1943). Similarly, Asperger described *"particularly interesting and highly recognizable"* type of children in his report. Despite wide individual differences, the common characteristics he observed also included *difficulties of social integration* and in *learning simple practical skills*, however sometimes *high level of original thought and experience* and ability to express themselves in a *linguistically original form* (Asperger, 1944; Asperger and Frith (Trans), 1991) [‡]. Although both reports have the common denominator on the behavioral phenotype and a neuropathological origin reasoned by the early onset, Asperger was

[†] Translated from the original quote by Eugen Bleuler (1911): *„Die schwerste Schizophrenen […] leben in einer Welt für sich; […] sie beschränken den Kontakt mit der Außenwelt so weit als möglich. Diese Loslösung von der Wirklichkeit zusammen mit dem relativen und absoluten Überwiegen des Binnenlebens nennen wir Autismus."*

[‡] Translation by Uta Frith (1991) summarizes Hans Asperger's description of symptoms and omits the discursive introduction and discussion of then current typologies.

the first to speak about a "genetic factor" possibly involved in the etiology of autism (Asperger, 1944; Sala and Verpelli, 2016).

Since the first seminal reports, the diagnostic criteria for autism have been reformulated several times. Our understanding of autism relies on the research in the past 70 years, which evolved exponentially since the mid-1990s (Lai et al., 2014). Today, autism is defined as a broad spectrum of neurodevelopmental conditions possessing a complex phenotype, namely Autism Spectrum Disorder (ASD). The revised diagnostic criteria for ASD in the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) are:

A. Persistent deficits in social communication and social interaction across multiple contexts
B. Restricted, repetitive patterns of behavior, interests or activities
C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities, or may be masked by learned strategies in later life).
D. Symptoms cause clinically significant impairment in social, occupational or other important areas of current functioning.
E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder), or global developmental delay.

The latest revision joins individuals who were previously – based on DSM-IV – diagnosed with autistic disorder, Asperger's disorder, or pervasive developmental disorder not otherwise specified, under the diagnosis of ASD (American Psychiatric Association, 2013). The new criteria thereby cover a wider range of individuals carrying the key symptoms, emphasizing the broad nature of ASD (Lai et al., 2014).

Already in the first publications by Kanner (1943) and Asperger (1944), there was a clear sex bias evident in children carrying autistic features. Current statistics as well indicate that ASD is strongly biased towards males, with a ratio of 4:1 (Gillberg et al., 2006; Werling and Geschwind, 2013), however the explanation for higher male prevalence remains unknown (Baron-Cohen et al., 2009, 2011). Presence of a female protective effect has been speculated, such that a component of female sex can protect girls from ASD, requiring a greater inherited genetic load for them to display autistic behavioral impairments (Robinson et al., 2013). This hypothesis was tested by Jacquemont et al. (2014), showing that clinical manifestations of neurodevelopmental

disorders, including ASD, require a higher mutational burden for females, supporting the protective effect in females. Furthermore, ASD in females, especially those with high-functioning ASD, might be under-diagnosed, or diagnosed at a later age, leading to a diagnostic bias. X- and Y-chromosome theories and fetal testosterone model, amongst others, are theories that may possibly lead to a gender bias, which however need further empirical support (Baron-Cohen et al., 2011).

Recent epidemiological research on ASD points out a prevalence of 0.76% to 2.6% (Lai et al., 2017), a ratio which has been increasing since the first epidemiological report (Lotter, 1966). Although growing risk factors cannot be ruled out, the increased incidence might partially be due to many other factors, such as "diagnostic switching" allowing decreased age of diagnosis, as well as availability of screening services and improved public awareness (Fombonne, 2009). One aspect which is affected by the changes in diagnostic criteria is the growing evidence that, the increase in prevalence rates can also be partially accounted by the comorbidity of intellectual disability (ID) and ASD. Hence, children initially diagnosed only with ID, could now be falling into ASD categories (Matson and Shoemaker, 2009). On the contrary, there is also the speculation that due to specific exclusionary criteria in DSM-5, individuals with ID, including those with specific genetic etiologies, may be underrepresented in ASD research (Dykens and Lense, 2011). Comorbidity of ID and ASD is consistently observed in many studies (Matson and Shoemaker, 2009; Matson and Cervantes, 2013). Although earlier statistics (DSM-IV-TR) notes a co-occurrence rate of up to 75%, this estimate might no longer be true due to reasons mentioned above (Dykens and Lense, 2011). Nevertheless, the current DSM indicates that about 70% of individuals with ASD may have one comorbid mental disorder, and 40% may have two or more (American Psychiatric Association, 2013). Epilepsy, attention-deficit hyperactivity disorder (ADHD), and anxiety, among other psychiatric and medical conditions, are reported to coexist with ASD (Amiet et al., 2008; Gillberg, 2010; Mannion and Leader, 2014). Along with the aforementioned comorbidity, Rett syndrome, fragile X syndrome (FXS), and tuberous sclerosis (TSC) are conditions that are associated with or carry symptoms of ASD (Folstein and Rosen-Sheidley, 2001; Hagerman et al., 2011; Neul, 2011).

Environmental exposures have been under exclusive focus as causative, and as contributory to increased incidence in ASD. Relationship between advanced parental age and ASD have been reported in numerous studies (Reichenberg et al., 2006; Croen et al., 2007; Durkin et al., 2008).

A genome-wide sequencing study has shown that fathers transmit a much higher number of mutations to their offspring than the mothers, being the dominant factor in determining the number of *de novo* mutations in the child. These mutations transmitted increase at a rate of about two mutations per year, implicating the importance of father's age as a risk factor in diseases, such as schizophrenia and ASD (Kong et al., 2012). Prenatal or perinatal infections due to exposure to viral or bacterial agents have also been associated with ASD (Yamashita et al., 2003; Atladóttir et al., 2010) reflecting the importance of immune involvement (Patterson, 2009, 2011; Brown, 2012). Furthermore, prenatal exposure to valproic acid (VPA), a clinically used agent in epilepsy and as a mood stabilizer in bipolar disorder treatment, is one other environmental factor that contributes to ASD incidence (Christianson et al., 1994; Rasalam et al., 2005; Christensen et al., 2013).

While environmental factors are widely associated with ASD etiology, there is now growing evidence that ASD also has a genetic basis (Abrahams and Geschwind, 2008). In fact, most of the aforementioned environmental risk factors as well exert their effect on ASD genesis via molecular or epigenetic modifications (Grabrucker, 2013; Carbonetto, 2014).

1.1.1 Genetics of ASD

The exact neurobiological causes of ASD remain largely undiscovered; however, several data have consistently shown a high genetic contribution to disease etiology. The first and utmost evidence came from studies indicating the high concordance rate for ASD in monozygotic twins (Folstein and Rutter, 1977; Bailey et al., 1995). In addition, the concordance rates for ASD are reported to be higher among monozygotic twins in comparison to dizygotic twins (Bailey et al., 1995; Lichtenstein et al., 2010). Further epidemiological studies focusing on concordance rates in twins and in families provided crucial information about the heritability of ASD, however these do not disclose the underlying genetic factors. Whole-genome analyses, as well as research on candidate genes performed in the last decade provide us with the relevant information for a better understanding of the pathophysiology underlying ASD (Huguet et al., 2016).

Human genome is highly diverse, i.e. there is an immense genetic variation among individuals and between populations (Cann, 1998). Recent consortium on the Human Genome Project, having reconstructed the genomes of 2,504 individuals from 26 populations, reports that an individual carries 4.1 million to 5 million genetic variants that differ from the reference human genome. These variants are mainly single-nucleotide polymorphisms (/variants) (SNP/SNV), short insertions/deletions (indels), and structural variants such as copy-number variants (CNV) (Bourgeron, 2015; Auton et al., 2015). These CNV may exert their effect on gene expression directly by disrupting genes and altering dosage or indirectly through a position effect or unmasking of recessive mutations or functional variants on the remaining allele in the case of a deletion (Lamb, 2011). Sebat et al. (2007) tested the hypothesis that *de novo* CNV are associated with ASD. Using comparative genomic hybridization, a method that allows detection of variations in DNA copy number of test samples relative to reference samples (Pinkel and Albertson, 2005), *de novo* CNV were shown to be significantly associated with ASD, affecting 10% of the patients with sporadic (simplex) ASD and 3% of familial (multiplex) cases. This as well highlights the higher risk to have a child with ASD in families in which a sibling is affected (Jorde et al., 1991; Sandin et al., 2014). One further remark of the study is the difference between frequency of *de novo* variants between simplex and multiplex cases, pointing out the possibility that distinct mechanisms may be involved in each (Sebat et al., 2007; Abrahams and Geschwind, 2008). In the largest and most comprehensive genomic analysis of ASD conducted to date, *de novo* CNV from 2,591 families were analyzed. Overall a strong evidence for association of *de novo* mutations with ASD apart from the risk for ID was found, replicating prior findings on a larger scale (Sanders et al., 2015). Other numerous studies published in the past decade (e.g. Neale et al., 2012; O'Roak et al., 2012) highlight the importance of *de novo* mutations in ASD, while at the same time underscoring the complexity of the disorder (McClellan and King, 2010).

One major challenge ASD faces regarding genetic research is the heterogeneity of the disease. ASD has a complex genetic architecture in which variants in over hundred genes may contribute to its etiology, in contrast to Mendelian disorders, in which a small number of genes lead to a phenotype (Sanders, 2015). While in some patients ASD can be caused by a single fully penetrant mutation, in another it might be by the accumulation of many low-risk alleles (Huguet et al., 2013; Bourgeron, 2015). Large CNV, for example, were found likely to contain multiple modest-effect risk genes (Sanders et al., 2015). Furthermore, not all individuals with these variants have ASD (incomplete penetrance), where the severity of ASD may vary in those who

do (variable expressivity) (Sanders, 2015). Moreover, several risk loci identified are related to more than one psychiatric or neurocognitive phenotype, so called pleiotropy (Cook Jr and Scherer, 2008).

Whereas tremendous research on genetics has been made in the field of ASD, epigenetic mechanisms are yet to be unveiled. Epigenetics by definition refer to the changes in any process that alters gene activity in the absence of a change in DNA sequence, leading to modifications in gene expression that are heritable. Several epigenetic modifications have been identified, which include methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation. DNA methylation, which switches off gene transcription, and histone acetylation leading to transcriptional activation fall into most common examples. Epigenetic modifications are naturally essential for many organism functions, however can have adverse effects if they occur improperly (Weinhold, 2006). A wide variety of diseases are linked to epigenetic processes, including neurodevelopmental disorders. For example, involvement of regulatory epigenetic mechanisms in developing ASD-like symptoms is profoundly evident in Rett syndrome and FXS pathogenesis. Rett syndrome is an X-linked disorder caused by mutations in the gene encoding the methyl CpG-binding protein 2 (MeCP2), a protein that is essential for postnatal brain development. MeCP2 selectively binds to methylated CpG (cytosine-phosphate-guanine) residues and mediates transcriptional repression of methylated constructs. The mutations leading to a malfunction of the MeCP2, hence to disease pathogenesis, point out the crucial role of epigenetic regulation (Amir et al., 1999). FXS likewise is associated with defects in epigenetic control mechanisms. FXS is caused by loss-of-function mutations in *FMR1* gene, which leads to an anomaly rendering the CGG repeat of the *FMR1* gene susceptible to epigenetic modifications and silencing (Schanen, 2006; Tabolacci and Chiurazzi, 2013). Further evidence, pointing epigenetic factors as causative and linked to ASD are the hotspots subject to imprinting – the condition where either of the maternal or the paternal allele is silenced by an epigenetic process. Prader Willi syndrome and Angelman syndrome are well-known examples of one such mechanism (Schanen, 2006).

Overall, ASD can be attributed to genetic alterations in 10-15% of the identified cases, with the larger fraction of cases still awaiting a causal link (Spooren et al., 2012). The challenge in the genetic research lies in making sense of the large number, and diverse nature of the genes associated with ASD, such as identifying the convergent molecular pathways (Ebert and

Greenberg, 2013). Recent studies have shown that many of the susceptibility genes converge in pathways that control synaptic functions (Ebert and Greenberg, 2013; Bourgeron, 2015). The research on the molecular basis of ASD is therefore immensely focused on disturbances of synaptic homeostasis in recent years.

1.1.2 Synaptic Pathways and ASD

Synapse, the site at which two neurons communicate and allow information transmission throughout the brain, is the key component of neurons. This continuous flux of information is organized into perception, learning and memory, and appropriate behavioral responses (Kandel, 2000). Therefore, a proper synaptic communication is crucial for the physiological processes to occur in the brain, and perturbations in this circuitry can lead to a variety of psychiatric disorders (Lepeta et al., 2016). The chemical synapses are highly specialized neuronal structures, comprising presynaptic and postsynaptic compartments, coming into contact through the synaptic cleft. Presynaptic bouton consists of neurotransmitter-filled synaptic vesicles, and the active zone, i.e. where the neurotransmitter release takes place. Postsynaptic compartment harbors the neurotransmitter reception and transduction of the received signal into electrical and biochemical changes through the finely tuned machineries. Presynaptic and postsynaptic sites are physically held together via the cell adhesion molecules (CAMs). CAMs linked to cytoskeleton not only generate stable synapse connection, but also, by conferring a flexible structure, enable plasticity in synapse shape and size (Benson and Huntley, 2012). Perhaps the best-characterized CAMs are the postsynaptic neuroligins (NLGNs) and their presynaptic binding partners; neurexins (NRXNs). Their interaction is likely to affect synaptic transmission, and their dysfunction can impair the properties of synapses and disrupt neural networks (Südhof, 2008).

The signal transduction in the chemical synapse is either excitatory or inhibitory, based on the specialization of the receptors either for glutamate or γ-aminobutyric acid (GABA), respectively. In mammalians, excitatory synapses mostly occur on the dendritic spines, and at the tip of the dendritic spines lies the morphologically and functionally specialized membrane called the postsynaptic density (PSD) (Sheng and Kim, 2011). PSD is highly complex containing hundreds

of proteins assembled from cytoskeletal and associated elements, membrane proteins (such as receptors, ion channels, and CAMs), signaling enzymes, as well as scaffold, anchoring, and adaptor proteins (Ryan and Grant, 2009; Sheng and Kim, 2011). Synapthopathy (or as some favor synapsopathy) is a term that has been increasingly used in the recent years, referring to disruptions in the synaptic structure and function (Brose et al., 2010). There is now emerging evidence that dysregulation of the synaptic activity may be a key component of the molecular basis of ASD. The fact that most of the ASD symptoms are observed within the first years of development – a period that coincides with the maturation/elimination of excitatory synapses and development of inhibitory synapses – points to the hypothesis that ASD might be a result of imbalance between excitation and inhibition in the developing brain (Ebert and Greenberg, 2013).

Glutamate, excitatory synaptic transmitter in the brain, can bind to ionotropic and metabotropic receptors. Three major ionotropic glutamate receptors are AMPA, NMDA, and kainate receptors, each of which named after the agonists that activate them, i.e. α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, *N*-methyl-D-aspartate, and kainate, respectively. Ionotropic receptors mediate Na^+ and K^+ flux, leading to membrane depolarization. While AMPA receptors are involved in fast excitatory neurotransmission, NMDA receptors, aided by the cofactor glycine, conduct signals only if both postsynaptic membrane depolarization and the binding of glutamate to the receptor occur, leading to coincidence detection and synaptic plasticity. One further exceptional property of the NMDA receptor is that, it controls a channel that allows Ca^{2+} influx in addition to Na^+ and K^+. NMDA receptors thereby are able to induce further cascades in the postsynapse that are related to calcium-dependent signaling, such as the calcium-dependent kinase (CaMK) and Ras–mitogen-activated protein kinase (MAPK) pathways. Binding of glutamate to the metabotropic glutamate receptors (mGluR) triggers protein-synthesis-dependent forms of signaling cascades via activation of secondary messengers (Kandel and Siegelbaum, 2000; Ebert and Greenberg, 2013). Glutamate receptors are connected to downstream signaling by directly binding the PSD-95 (postsynaptic density protein of 95 kD molecular weight, also known as SAP90) family of scaffolding proteins (Kandel and Siegelbaum, 2000; Sheng and Kim, 2011). PSD-95, along with several other PSD proteins, contains a PDZ domain (named after the first three proteins sharing the domain, i.e. PSD-95, disc large tumor suppressor (Dlg1) in *Drosophila*, and zonula occludens (ZO)-1), which allows interaction with

other proteins. Direct binding of PDZ domains to NMDA receptors allows PSD-95 to stabilize these receptors at the cell surface, and facilitates the NMDA receptor-associated downstream signaling cascades. Furthermore, by binding to accessory subunits of AMPA receptors, PSD-95 recruits AMPA receptors to the synapses, appearing to determine the size and strength of excitatory synapses (Kim and Sheng, 2004; Sheng and Kim, 2011).

The PSD contains several other scaffolding proteins, with SHANK (or ProSAP), SAPAP (or GKAP), and HOMER constituting the fundamental members of the scaffold complex. Primarily, SHANK interacts with (i) SAPAP via its PDZ domain, which in turn binds to PSD-95, hence indirectly to AMPA and NMDA receptors; (ii) HOMER, the protein that interacts with mGluR, (iii) NLGN, that holds the pre- and postsynaptic connection through interaction with NRXN, and (iv) actin filaments, that are crucial determinants of the excitatory synapse morphology. SHANK proteins therefore lie in the "epicenter" of the PSD, having the crucial role of holding and crosslinking various receptors and cytoskeletal elements (Naisbitt et al., 1999; Monteiro and Feng, 2017). Remarkably, several studies have shown that mutations in genes expressing PSD proteins, such as SHANK (Durand et al., 2007; Berkel et al., 2010; Sato et al., 2012) and NLGN (Jamain et al., 2003) confer a risk for developing ASD.

1.2 SHANK Family of Proteins

SHANK proteins are the "master" scaffolding proteins of the PSD, connecting neurotransmitter receptors and other membrane proteins with downstream signaling cascade and actin cytoskeleton. The name is derived from the SH3 (Src homology 3) domain and the multiple ankyrin repeats (ANK) they contain, in addition to the other domains that are essential for various protein-protein interaction, i.e. PDZ domain, proline-rich region (PRO), and sterile alpha motif (SAM) domain (Naisbitt et al., 1999). The N-terminal ANK domain of SHANK probably allows interaction with the cytoskeleton through binding to another PSD protein SHARPIN. The PDZ domain interacts with SAPAP and the AMPA receptors, while SAPAP can bind to PSD-95 which in turn interacts with NMDA receptors. PRO domain of SHANK binds to mGluR-interacting HOMER, as well as cortactin proteins, which interact with actin. SAM domain on the other hand

is required for the localization at the PSD. Together, this dense scaffold serves as an interface between the membrane-bound receptors, CAMs, and actin cytoskeleton (Boeckers, 2006). The significant role of SHANK proteins is therefore to tether and organize intermediate scaffolding proteins, hence they are crucial for proper synaptic development and transmission (Monteiro and Feng, 2017).

SHANK protein family has three known members: SHANK1, SHANK2, and SHANK3. The genes encoding these proteins have previously been cloned under different guises and were therefore also known under different names/abbreviations, including Synamon, SSTRIP, and ProSAP (Sheng and Kim, 2000). SHANK proteins are strongly expressed in the central nervous system, with individual isoforms being expressed at lower levels in the periphery including kidney, heart, spleen, liver (Lim et al., 1999), and cochlea (Braude et al., 2015). Alternative splicing mechanisms due to presence of alternative promoters and exons give rise to different isoforms in each *SHANK* gene (Lim et al., 1999). *SHANK1* gene in humans is located on chromosome 19q13.33 spanning 55.1 kb. It contains 23 exons and two alternative promoters leading to two isoforms, i.e. *SHANK1A* and *SHANK1B*. The long isoform *SHANK1A* contains all ANK, SH3, PDZ, PRO, and SAM domains, whereas *SHANK1B*, the shorter isoform, only consists of PDZ and SAM domains. *SHANK2* gene, located on chromosome 11q13.3 spanning 621.8 kb, contains 25 exons, with three alternative promoters and one alternative stop codon which give rise to four isoforms. *SHANK3* gene is located on chromosome 22q13.3, spans 55.1 kb, and contains 24 exons and one alternative stop codon leading to three isoforms (Leblond et al., 2014; Guilmatre et al., 2014).

Much of the information on SHANK expression pattern comes from the studies in rodents. In the mouse, *Shank* genes encoding the SHANK proteins also have alternative promoter regions subject to alternative splicing mechanisms, giving rise to several different isoforms (Lim et al., 1999; Sheng and Kim, 2000). For *Shank1*, *Shank2*, and *Shank3* the number of different transcripts identified to date, that are produced by intragenic promoters or alternative splicing exons, count to 2, 3, and 10, respectively (Monteiro and Feng, 2017). In the central nervous system, the three genes' mRNAs are differentially expressed. *Shank1* mRNA is highly enriched in the cortex, thalamus, amygdala, CA1 and CA3 regions of the hippocampus, dentate gyrus, and Purkinje cell layers of the cerebellum. *Shank2* mRNA is enriched in the cortex, CA1 and CA3 regions of the hippocampus, and Purkinje cell layers of the cerebellum. *Shank3* mRNA is widely

enriched in the striatum, thalamus, CA3 region of the hippocampus, and granule cells of the cerebellum (Böckers et al., 2004; Peça et al., 2011). Although all *Shank* transcripts were detected in the neuropil layer of the CA1 region, *Shank1* mRNA was found to have the highest dendritic expression among three homologues (Epstein et al., 2014). This enrichment in the neuropil, as compared to somata, is viewed as evidence for local translation to be an essential source of SHANK protein at the synapse during activity-dependent plasticity (Sala et al., 2015). Böckers et al. (2004) further showed that *Shank1* and *Shank2* mRNAs are highly expressed early in postnatal brain, whereas *Shank3* mRNA expression increases during postnatal brain development.

1.2.1 Shankopathies in ASD

Shankopathies were first demonstrated in neurodevelopmental disorders by studies of Phelan-McDermid syndrome (PMS) or also known as 22q13.3 deletion syndrome. As implied by the name, this syndrome results from the loss of the distal long arm of chromosome 22, the locus encoding *SHANK3* gene (Wilson et al., 2003). The deletion results in neurological deficits, including developmental delay, absent or delayed speech, and moderate to severe intellectual impairment. Remarkably, more than 50% of the patients exhibit ASD-like phenotype, hence the syndrome can be classified as a syndromic form of ASD (Phelan and McDermid, 2012). This finding was the first hint pointing the dysfunction of SHANK at the expense of developing ASD-like symptoms, and led to a further focus on the family to elucidate the pathogenesis. A meta-analysis study showed that mutations or disruptions in the *SHANK* gene family account for ~1% of ASD cases (Leblond et al., 2014).

Apart from the syndromic PMS form of ASD, many further *SHANK3* mutations/deletions have been identified in patients with ASD since the first description by Durand et al. (2007). These mutations/deletions encompassing various regions of the *SHANK3* gene were either *de novo*, inherited from an affected or unaffected mother or father, or of unknown origin in some cases. Common characteristics of the patients included severe ID, absent or delayed verbal speech, impaired social interaction, as well as motor stereotypies, repetitive behaviors and narrow interests (Moessner et al., 2007; Gauthier et al., 2009; Boccuto et al., 2012; Leblond et al., 2014),

or a schizophrenia phenotype (Gauthier et al., 2010). Mutations in *SHANK2* have been linked to ASD in a study screening several individuals with ASD, mental retardation, and unaffected healthy controls. The study identified *de novo* CNV in *SHANK2* gene in individuals with ASD and mental retardation, highlighting the common genes and a clinical overlap between the two disorders (Berkel et al., 2010). In parallel Pinto et al. (2010) also implicated mutations in *SHANK2*, along with other genes in patients with ASD, identifying novel pathways to ASD risk.

The first report on *SHANK1* mutations came to light in a study by Sato et al. (2012) assessing genetic screening data from 1,158 Canadian and 456 unrelated European individuals with ASD. A hemizygous deletion in *SHANK1* was identified in a four-generation family, in which male carriers but not female carriers were affected by the deletion. The males in the family were diagnosed with ASD with higher functioning, whereas the females carrying the deletion displayed no ASD-like symptoms but were rather diagnosed with anxiety disorder. In addition, a *de novo* deletion was also detected in an unrelated male individual with ASD with higher functioning. Surprisingly, there is relatively little variability among the affected individuals. Nonetheless, compelling evidence for a sex bias among the individuals carrying the *SHANK1* deletion was present (Sato et al., 2012). Sato et al. (2012) had postulated that *SHANK1* mutations might as well be linked to other brain disorders given that *SHANK2* and *SHANK3* mutations were observed also in ID and schizophrenia. In fact, in addition to ASD, mutations in *SHANK1* were linked to schizophrenia, and to ID in an individual with ASD. In a study screening almost 200 schizophrenia patients, the T-allele of the promoter variant rs3810280 of *SHANK1* gene was found to be significantly related to reduced auditory working memory capacity in schizophrenia patients and subjects clinically at risk for developing a psychosis (Lennertz et al., 2012). Pointing deficits in the synaptic networks, loss-of-function mutations in *SHANK1* were consistently found in another study screening individuals with schizophrenia or schizoaffective disorder (Fromer et al., 2014). Very recently, among a cohort screening over 1000 individuals with ASD, a *de novo* mutation in *SHANK1* was identified in a female presented with ID in addition to ASD (Wang et al., 2016a).

1.3 Animal Models of ASD

The complex and heterogeneous architecture of ASD is a challenging factor in uncovering the exact biological mechanisms underlying the autistic behaviors. The immense contribution of genetic factors and lack of reliable biomarkers make it tempting to develop animal models of ASD for a deeper understanding of the disease pathogenesis. Animal models of neuropsychiatric disorders are usually designed with regard to three criteria as proposed by Willner (1984), i.e. (i) face validity, (ii) construct validity, and (iii) predictive validity. Face validity refers to the resemblance of the model to the symptoms observed in humans. In case of ASD, for example, this expects the model to manifest the core symptoms used in diagnostics. Construct validity, on the other hand, involves the similarity of the mechanism underlying the disease, e.g. an animal carrying the mutation in the same gene as in the humans. Finally, predictive validity tests the ability of the model to respond to the treatments that are effective in humans (Willner, 1984; Crawley, 2004). In the recent years, there has been further propositions for refining the standards, including homological (strain and species basis), pathogenic (ontopathogenic and triggering-factor resemblance), and mechanistic (identical cognitive and biological mechanisms) validity as additional criteria (Belzung and Lemoine, 2011).

1.3.1 Testing ASD in Animal Models

Today, the diagnostic criteria for ASD are still purely behaviorally defined (American Psychiatric Association, 2013), and an in-depth genetic screening is a challenging factor due to heterogeneity of the disorder. Developing a mouse model that meets the validity criteria plays a crucial role to advance our understanding, thus behavioral phenotyping of the mouse model is a key component of the current translational approach. There is growing need for sensitive mouse behavioral test paradigms with high relevance to each diagnostic symptom category, namely deficits in social communication and interaction across multiple contexts, and repetitive patterns of behavior (Crawley, 2004). Given the merge in diagnostic symptoms of ASD regarding qualitative impairment in social interaction and qualitative impairments in communication,

current methods consider assessing these aspects simultaneously (Ricceri et al., 2016). In the hope of discovering the deficits and therapeutic targets, several assays have been developed that recapitulate the core symptoms and that constitute state-of-the-art behavioral phenotyping today (Crawley, 2004, 2007; Silverman et al., 2010; Wöhr and Scattoni, 2013; Pasciuto et al., 2015; Ricceri et al., 2016).

Social interaction

Mice are highly social species and live in group territories. Their intricate nature of social organization can therefore be tested observing their social behavior, such as social exploration, reciprocal social interaction, and social preference skills, as well as their territorial, parental, and sexual behavior. Using assays that can measure disturbances in this complex set of behaviors may be analogous to those of individuals with ASD, which corresponds to the diagnostic criterion of ASD (American Psychiatric Association, 2013). Several behavioral assays have been developed for qualitative assessment of the social behavior that can reveal ASD-like social abnormalities in mice (Crawley, 2004; Silverman et al., 2010).

Reciprocal social interaction can be assessed in specific environments, typically in a standard cage, in order to assess the social behavior of two unfamiliar mice, such as by means of sniffing (facial/anogenital), following each other, and allogrooming (Terranova and Laviola, 2005). Reduced interest in the partner and/or a delay to engage in the first contact can speak for a deficit in the task as seen in several ASD models (Panksepp et al., 2007; McFarlane et al., 2008; Peça et al., 2011). Dyadic interactions can also be tested by means of social approach test. The three-chambered box social approach test is the most sensitive test developed so far to assess levels of sociability in mice (Moy et al., 2004; Yang et al., 2011). In this task, a subject mouse, while freely exploring the apparatus, has the choice between spending time with a social stimulus, i.e. a stranger mouse that is constrained in one chamber, or with a non-social stimulus i.e. an object located in the other chamber. As mice are social animals, they would normally show interest in a stranger and explore the chamber where it is located. Spending equal or more time with the object indicates reduced sociability in the subject mouse, which resembles the social deficits in individuals with ASD – such as lacking interest in playing with other children, but engaging in

non-social activities instead. Same apparatus can also be used for studying social recognition by presenting a familiar stimulus mouse used during the social approach task and an unfamiliar novel mouse. Typically, mice prefer exploring the novel mouse (Crawley, 2004; Silverman et al., 2010; Wöhr and Scattoni, 2013). One drawback of the three-chambered social approach/recognition task is that it allows measuring the social activity initiated by the subject mouse only, thus fewer details of reciprocal interactions are captured. Nonetheless, it is a standardized tool for analysis of social behavior, which was able to assess reduced sociability in several ASD models of interest (Tabuchi et al., 2007; Peça et al., 2011; Won et al., 2012). Social transmission of food preference is another assay with potential face validity to the symptoms of ASD (Crawley, 2004; Ryan et al., 2008), that was initially developed as a memory task (Galef and Wigmore, 1983). In this paradigm, a subject mouse interacts with a demonstrator cagemate to form a food preference based on cues transferred from the demonstrator. The task therefore requires a successful social communication between the two mice, which appears to fail in models of ASD (McFarlane et al., 2008).

Communication

Delayed or absent verbal speech would be a hint depicting communication deficits in humans with ASD. However, unlike in humans, testing communication deficits in mice is a challenging task, as means of communication in this species is different than that of humans. Mice, instead, communicate using olfactory social signals (Arakawa et al., 2008) and emitting ultrasonic vocalizations (USV) in a variety of situations (Branchi et al., 1998, 2001; Wöhr and Schwarting, 2010). Measuring USV is therefore a useful tool to bring about the communication deficits in mouse models. In fact, alterations in ultrasonic communication has been reported in several mouse models of neurodevelopmental disorders (e.g. Scattoni et al., 2008, 2009; Jamain et al., 2008; Wöhr et al., 2011).

Perhaps the most extensively studied branch of mouse USV is the neonatal USV as an indicator of the early communicative capability. These USV emitted by the pups were first discovered by Zippelius & Schleidt (1956), in a situation where the pups were separated from their mothers and littermates. Under this condition, mice were observed to emit calls with

frequencies up to 80 kHz. Isolation-induced USV serve a communicative function, such that the mothers leave the nest, search and retrieve the pups which were scattered outside the nest. This retrieval behavior of the mothers appears to be specific to vocalizing pups, as no such behavior was present in response to anesthetized or sacrificed pups. Moreover, by means of playback experiments Sewell (1970) showed that mothers respond solely to USV emitted by the pups, but not to background noise or artificial pulses of 45 kHz frequency, indicating the significance of this type of communication.

Pup USV typically follow an ontogenic profile, i.e. they peak at around eighth day after birth and decrease to zero levels at around 2 weeks of age, following an inverted U-shaped pattern (Noirot, 1966; Elwood and Keeling, 1982). They occur at frequencies between 30 kHz and 90 kHz; commonly named "60-kHz USV" (Wöhr and Schwarting, 2010). Based on their internal pitch changes, lengths, and shapes, these USV were classified into distinct categories (Scattoni et al., 2008). Albeit the restricted knowledge about the function of such different waveform patterns, it was shown that mothers are able to distinguish between different pup USV categories based on call features. They prefer certain call characteristics over others, indicating that acoustic parameters, such as call duration, peak amplitude, and peak frequency, affect the functional value of isolation-induced USV(Sewell, 1970; Smith, 1976; Ehret and Haack, 1982; Wöhr et al., 2008).

Juvenile mice as well emit USV, during social investigation of a conspecific – so called "interaction-induced USV" or "70-kHz USV" (Panksepp et al., 2007; Wöhr and Schwarting, 2010). These USV appear to be specific to the social interaction *per se*, as the USV production was found to be significantly correlated with the investigation responses of the mice (Panksepp et al., 2007). In adulthood, USV production can occur during female-female interactions, or during male-female interactions such as in mating behavior (Sewell, 1967). These USV, possibly inducing receptive behavior in females, are emitted by the male during mating, as well as if the male is exposed to female urine. Hence, they were classified as "female-induced USV", which also appear at 70 kHz range. The main difference between juveniles' interaction-induced USV and adult males' female-induced USV is the lack of whistle-like character in the latter. Instead, they appear rather rough and noisy (Wöhr and Schwarting, 2010).

In mouse models of ASD, it is predicted that pups with ASD-like behavior will emit fewer USV, and/or USV with aberrant call characteristics (e.g. duration, frequency, amplitude, classification). From the maternal side, on the other hand, mouse models of ASD may fail to

respond to USV of the pups, which can be measured by deficits in retrieving the pups. Likewise, deficits in USV emission at later phases of development, which can be measured quantitatively, may as well yield insights into ASD-relevant communicative dysfunctions (Crawley, 2004; Wöhr, 2014).

Repetitive behaviors

Certain behavioral assays have been developed to study restricted, repetitive patterns of behavior and stereotypies. Stereotypy is defined as behaviors that are apparently purposeless and persist for unusually extended periods, such as hand flapping and head rolling. Mice as well can exhibit spontaneous motor stereotypies, including jumping, backflips, circling, digging, and excessive self-grooming (Lewis et al., 2007), with the notion that housing conditions of the laboratory animals *per se* can as well drive some of the stereotyped behavior (Garner and Mason, 2002). Lewis et al. (2007) have conceptualized mouse repetitive behaviors in two clusters: one being the "lower order" motoric actions with stereotyped movements and/or self-injury, while the "higher order" cluster being characterized by more complex behaviors that have a cognitive component, such as compulsions, repeated rituals, and insistence on sameness. In mice, repetitive behavior can be typically tested by placing a mouse in a cage without bedding to score the time spent self-grooming (McFarlane et al., 2008; Moy et al., 2008). Marble burying test could also reveal repetitive behavior by means of scoring marbles buried and time spent digging, not correlated with anxiety-related behaviors (Thomas et al., 2009). In rodents, burying can occur as a defensive action in response to aversive stimuli in rodents, which can be scorpions or insects in the wild, and air-puffs or shock electrodes in the laboratory conditions (Pinel and Treit, 1978; Thomas et al., 2009). However, objects that are not aversive or dangerous can also elicit burying response, such as food-pellets or glass marbles (Broekkamp et al., 1986; Thomas et al., 2009). There are a number of mouse models that exhibit intensive self-grooming, and/or elevated digging and abnormal marble burying behaviors which can be translated into ASD-like behavior in humans (McFarlane et al., 2008; Ryan et al., 2008; Peça et al., 2011). In addition to marble burying test, exploratory hole-board task, during which deficits in hole selectivity can reflect persistent, repetitive behaviors, has also been suggested as a strategy to model ASD-like phenotype in mice (Moy et al., 2008).

1.3.2 Shank1 Mouse Model for ASD

Creating *Shank* mouse models for ASD hold great promise for comprehending the underlying biological mechanisms *in vivo*, given that mutations in all *SHANK* variants were repeatedly reported in individuals with ASD (Moessner et al., 2007; Durand et al., 2007; Gauthier et al., 2009; Berkel et al., 2010; Pinto et al., 2010; Leblond et al., 2012; Sato et al., 2012). The first *Shank* model, i.e. *Shank1* mouse model for ASD, was introduced by Hung et al. (2008), which was followed by generation of *Shank2* (Schmeisser et al., 2012; Won et al., 2012) and *Shank3* mouse models for ASD (Bozdagi et al., 2010; Peça et al., 2011; Wang et al., 2011, 2016b; Schmeisser et al., 2012; Kouser et al., 2013; Lee et al., 2015; Speed et al., 2015; Zhou et al., 2016; Mei et al., 2016; Jaramillo et al., 2016).

Shank1 mouse model was generated via disruption of the *Shank1* gene through deletion of exons 14 and 15 (the region encoding PDZ domain), leading to a complete knockout of all SHANK1 proteins. The first phenotyping of the model revealed that *Shank1*$^{-/-}$ null mutants are grossly indistinguishable from their *Shank1*$^{+/+}$ wildtype littermates. They showed similar survival rates as the littermates. In general, *Shank1*$^{-/-}$ mice were poor breeders, giving birth only rarely. Furthermore, the *Shank1*$^{-/-}$ mothers did not nurture their pups, possibly causing the litters to generally die before weaning. Therefore, all studies in *Shank1*$^{-/-}$ mice were performed on offspring of *Shank1*$^{+/-}$ heterozygous crosses. Another advantage of heterozygous crossing is that, the breeding is expected to yield offspring that represents all three genotypes. The expected Mendelian ratio in the litters' genotypes was present after *Shank1*$^{+/-}$ breeding (Hung et al., 2008). No obvious physical differences were detectable between the genotypes (Silverman et al., 2011). At the neuroanatomical level, no gross abnormalities in the size or histological structure of the brain were detected. However, synapse morphology was found to be altered in the hippocampus of *Shank1*$^{-/-}$ mice. Specifically, smaller dendritic spines and decreased number of synapses, resulting in a weakening of excitatory synaptic transmission was observed in *Shank1*$^{-/-}$ mice (Hung et al., 2008). PSD protein composition was also altered in *Shank1*$^{-/-}$ mice, with reduced levels of SAPAP (GKAP) and HOMER, which are binding partners of SHANK1 protein (Hung et al., 2008).

Behavioral characterization revealed that *Shank1*$^{-/-}$ mice show increased anxiety-related behavior. Furthermore, they manifest impaired contextual but normal fear memory, with enhanced acquisition but impaired retention of spatial learning, possibly resembling the aberrant cognitive phenotype present in some ASD cases (Hung et al., 2008; Silverman et al., 2011). While social interaction and repetitive behavior was reported to be unchanged (Silverman et al., 2011), evidence for lifelong communication deficits was provided (Wöhr et al., 2011). Specifically, Wöhr et al., (2011) found reduced isolation-induced USV in pups, together with early developmental deficits. They further observed a lack of social modulation of adult male USV in response to female urine and reduced scent marking behavior in *Shank1*$^{-/-}$ mice, indicating a failure to learn from social experiences.

2 OBJECTIVES AND HYPOTHESES

Previous studies on the *Shank1* mouse model for ASD provide basic insights into behavioral phenotype of the model. In this dissertation, the main objective is to investigate ASD-related deficits in *Shank1* knockout mice by dissociating between social and non-social components throughout development, considering (i) neurodevelopmental nature of the disorder and (ii) this occurring through multiple contexts in humans with ASD.

In previous studies, incongruous results on social behavior and a mixed cognitive phenotype were reported in *Shank1* mutants (Hung et al., 2008; Silverman et al., 2011). Therefore, socio-cognitive processes and potential underlying mechanisms remain to be investigated systematically in *Shank1* mutant mice. For this purpose, in Study I, ASD-related phenotypes with particular emphasis on social behavior and cognition in *Shank1* mouse mutants in comparison to wildtype and heterozygous littermate controls were assessed across development. At the neurobiological level, expression of learning-associated hippocampal brain-derived neurotrophic factor (BDNF) and its epigenetic regulation were analyzed to find out whether alterations in BDNF potentially contribute to ASD-relevant phenotypes in *Shank1* mutants. As for socio-cognitive aspect, deficits in social behavior and cognitive impairments are expected in *Shank1* mutants, reflected by altered BDNF levels at the neurobiological level.

Communication deficits were previously reported in *Shank1* knockout mice by means of reduced isolation-induced USV in pups (Wöhr et al., 2011). However, it is not known whether these deficits are due to general impairment or delay in development. Furthermore, effects of social context on communication have not been reported yet. To answer these questions, Study II has focused on effects of development and social context on communication deficits, assessed by isolation-induced pup USV. Mice carrying *Shank1* deletion are expected to display a shift in USV emission pattern, and prominent differences under social context, in comparison to wildtype and heterozygous littermate controls.

Shank1 mutants, however also wildtype and heterozygous controls, were previously reported to engage in elevated levels of self-grooming, indicating the necessity for a further assessment of the repetitive behavior in *Shank1* mutants. Therefore, in Study III ASD-like repetitive behaviors

were assessed. By establishing a test paradigm that allows assessing the effects of social context, occurrence of repetitive behaviors were evaluated in a genotype-dependent manner. In that aspect, *Shank1* mutants, in comparison to their wildtype and heterozygous controls, are expected to manifest elevated levels of repetitive behaviors, especially when tested in a social context.

3 SUMMARY OF PUBLICATIONS

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders with a strong genetic component. Today, ASD is characterized by persistent deficits in social communication and interaction across multiple contexts, with restricted, repetitive patterns of behavior. Mutations in genes encoding SHANK postsynaptic family of proteins, namely *SHANK1*, *SHANK2*, and *SHANK3* genes have been repeatedly reported in individuals with ASD. As the current diagnostic tools for ASD depend purely on behavioral phenotyping due to lack of reliable biomarkers, there is a growing need for use of animal models to discover the mechanisms underlying the behavioral deficits. The aim of the studies presented here was to understand these mechanisms throughout development and under different social contexts, by using *Shank1* mouse model for ASD, with a focus on each diagnostic symptom, namely deficits in social behavior (Study I), communication (Study II), and repetitive behavior (Study III), as well as cognitive deficits (Study I). Applying state-of-the-art behavioral phenotyping approaches, these studies aimed to reveal any possible deficit in *Shank1* knockout mouse model that might be analogous to humans.

Study I: Aberrant cognitive phenotypes and altered hippocampal BDNF expression related to epigenetic modifications in mice lacking the post-synaptic scaffolding protein SHANK1: Implications for autism spectrum disorder

Sungur AÖ, Jochner MCE, Harb H, Kılıç A, Garn H, Schwarting RKW, Wöhr M. in press. Aberrant cognitive phenotypes and altered hippocampal BDNF expression related to epigenetic modifications in mice lacking the post-synaptic scaffolding protein SHANK1: Implications for autism spectrum disorder. **Hippocampus.**

Summary

Behavioral studies employing *Shank1*$^{-/-}$ null mutant mice reported increased anxiety-related behavior, impaired contextual fear memory, and enhanced acquisition but impaired retention of spatial learning, possibly resembling the aberrant cognitive phenotype present in some ASD

cases. Although previous reports led to the interpretation that they do not demonstrate ASD-relevant social interaction deficits, further validation of this test is demanded. In this study, ASD-related phenotypes, with particular emphasis on social behavior and cognition, were assessed in *Shank1*$^{-/-}$ mice in comparison to heterozygous and wildtype littermate controls across development in both sexes. To assess social approach, social recognition, and object recognition in *Shank1*$^{-/-}$ mice, the three-chambered box assay was used. While social approach behavior was evident in all experimental conditions and social recognition was only mildly affected by genotype, *Shank1*$^{-/-}$ mice were severely impaired in object recognition memory. This effect was particularly prominent in juveniles, not due to impairments in object discrimination, and replicated in independent mouse cohorts. At the neurobiological level, object recognition deficits were paralleled by increased brain-derived neurotrophic factor (BDNF) protein expression in the hippocampus of *Shank1*$^{-/-}$ mice; yet BDNF levels did not differ under baseline conditions. Therefore epigenetic regulation of hippocampal BDNF expression was further investigated, and enrichment of histone H3 acetylation was detected at the *Bdnf* promoter1 in *Shank1*$^{-/-}$ mice. As increased acetylation of a gene refers to transcriptional activation, this result appears to be consistent with increased learning-associated BDNF. Together, these findings indicate that *Shank1* deletions lead to an aberrant cognitive phenotype characterized by severe impairments in object recognition memory and increased hippocampal BDNF levels, possibly due to epigenetic modifications. This result supports the link between ASD and intellectual disability, and suggests epigenetic regulation as a potential therapeutic target.

Study II: Early communication deficits in the Shank1 knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context

Sungur AÖ, Schwarting RKW, Wöhr M. 2016. Early communication deficits in the Shank1 knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context. **Autism Research** 9:696–709.

Summary

SHANK1 deletion was recently found in individuals with ASD. Remarkably, *SHANK1* deletion leads to a relatively little variable expressivity, with affected males manifesting similar

communicative dysfunctions at the early stage of development. In this study, communication deficits were investigated by means of isolation-induced pup ultrasonic vocalizations (USV) in the *Shank1* mouse model for ASD. For that purpose, *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate controls were compared. The first aim of the study was to evaluate the effects of *Shank1* deletions on developmental aspects of communication in order to see whether ASD-related communication deficits are due to general impairment or delay in development. Here it could be shown that *Shank1*$^{-/-}$ pups vocalized less and displayed a delay in the typical inverted U-shaped developmental USV emission pattern with USV rates peaking on a later postnatal day as compared to *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ littermate controls, resulting in a prominent genotype difference. Furthermore, USV emitted by *Shank1*$^{-/-}$ pups had lower amplitudes, as compared to *Shank1*$^{+/+}$ controls, possibly leading to *Shank1*$^{-/-}$ pups being less efficient in attracting mothers and inducing maternal care. In a second experiment, effects of social context on USV production were sought. When the pups were tested under social context, genotype-dependent deficits seen in first experiment were even more prominent, regardless of the familiarity of the social odor. As communication by definition serves a social function, introducing a social component to the typically nonsocial test environment could help revealing communication deficits in mouse models for ASD. Together, results of this study indicate that *SHANK1* is involved in acoustic communication across species, with genetic alterations in *SHANK1* resulting in social communication/interaction deficits.

Study III: Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context

Sungur AÖ, Vörckel KJ, Schwarting RKW, Wöhr M. 2014. Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context. **Journal of Neuroscience Methods** 234:92–100.

Summary

Restricted, repetitive patterns of behavior, interests or activities constitute the second diagnostic criteria of Autism Spectrum Disorder (ASD). Mice as well can show stereotypies, which can be evaluated using sensitive behavioral paradigms. Typically, repetitive behavior in mouse models for ASD is assessed by measuring self-grooming and/or digging behavior. In this

study, repetitive behaviors in *Shank1*$^{-/-}$ null mutant mice were assessed at juvenile and adult age, by comparing *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice by means of a comprehensive and sensitive approach, including the assessment of self-grooming, digging behavior, and marble burying. In order to establish a test paradigm that allows assessing the effects of social context on the occurrence of repetitive behaviors in a genotype-dependent manner, repetitive behaviors were repeatedly tested on three consecutive days under distinct social contexts, achieved through presence or absence of social odors. Our analyses showed that *Shank1*$^{+/-}$ and to a lesser extent *Shank1*$^{-/-}$ displayed slightly elevated levels of self-grooming behavior as adults, but not as juveniles, with genotype differences being most prominent under social context. Furthermore, marble burying was strongly reduced in adult *Shank1*$^{+/-}$ and *Shank1*$^{-/-}$ mice across social contexts, as compared to adult *Shank1*$^{+/+}$ littermate controls. These results support the notion that, effects of *Shank1* deletion on repetitive behaviors are influenced by development and social context, and further in line with a number of studies with other genetic *Shank* models.

4 GENERAL DISCUSSION

Since the first mutation described in humans (Durand et al., 2007), *SHANK* gene family became one of the most promising candidate set of genes for ASD (Persico and Bourgeron, 2006; Abrahams and Geschwind, 2008; Leblond et al., 2014; Guilmatre et al., 2014), and several *Shank* mouse models were generated in order to investigate the contribution of *SHANK* genes to ASD. Findings from these models, each of which to a certain extent reflect the symptoms observed in affected individuals, have shed a light on understanding the fundamental pathology (Yoo et al., 2014; Wöhr, 2014). Through three studies, this dissertation aimed at investigating ASD-like behavioral phenotypes and cognitive functions of *Shank1* knockout mouse model, along with a comprehensive focus on each category of ASD diagnostic symptom throughout development and under different social contexts to establish a translational link across species. To this end, in the following sections I will firstly discuss effects of development and social context on core ASD deficits in *Shank1* knockout mouse model for ASD. Next, I will focus on the most common comorbid condition in ASD, namely ID, and expand this in terms of cognitive deficits observed in *Shank1* knockout mice. To provide insights into ASD pathogenesis, I will then give an overview on neurobiological findings, and approach the model from a translational perspective to conclude on a reasonable analogy.

4.1 Shank1 Deletion Leads to ASD-Like Behavioral Phenotypes throughout Development

Deficits in social communication and interaction, and restricted, repetitive patterns of behavior constitute the clinical diagnostic criteria of ASD. In studies I-III, these core symptoms were investigated in *Shank1* mouse model for ASD throughout development – from neonatal to juvenile phase and in adulthood – in order to evaluate persistence of deficits. Evidence for strong effects of development on key diagnostic components was found in *Shank1* knockout mice. First of all, social behavior assessment by means of social approach and social recognition paradigms revealed major genotype differences in an age-dependent manner. Specifically, results from

Study I indicate that, while *Shank1* mutants showed normal social approach behavior at all developmental phases, i.e. interacting with a conspecific instead of exploring an object when given the choice, social memory was impaired during adulthood but not at juvenile age. In addition to social behavior, communication deficits stand out as another core symptom to be affected by development. In Study II, developmental aspects of ultrasonic communication in the *Shank1* mouse model were sought by means of isolation-induced pup USV, and interaction-induced USV during social approach. Notably, *Shank1*$^{-/-}$ mutant mice emitted fewer USV when isolated from the mother and littermates, depicting a general communication deficit, as previously reported (Wöhr et al., 2011). In addition, this general communication deficit was clearly reflected by a delay in development, as *Shank1*$^{-/-}$ mutant pups displayed a shift in their call pattern with USV rates peaking later than *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{+/+}$ wildtype littermates. At juvenile age, however, no deficits in USV emission were observed, with mice of all genotypes vocalizing in the chamber where a social stimulus is present. Finally, effects of development on restricted, repetitive patterns of behavior were investigated in Study III. While self-grooming behavior was not different between genotypes in juvenile mice, this pattern was towards an increased repetitive behavior assessed by self-grooming in adult *Shank1*$^{-/-}$ mutant mice. Therefore, through deletion of *Shank1*, deficits in social behavior, communication, and repetitive behavior appear to progress with development, reflecting the neurodevelopmental nature of ASD.

Previous information on social behavior of juvenile and adult *Shank1* mice relies on the studies by Silverman et al. (2011) and Wöhr et al. (2011). Silverman et al. (2011) assessed social behavior by means of reciprocal social interaction in juvenile mice, and social approach task in three-chambered box assay during adulthood. While this study indicated normal social behavior at both developmental phases, Wöhr et al. (2011) reported deficits in social behavior, where adult *Shank1*$^{-/-}$ male mice failed to learn from social experiences. Likewise, in Study I, strong deficits in social recognition were revealed both in adult females and males, despite lack of social novelty in *Shank1*$^{+/+}$ control males. The ability of all *Shank1* genotypes to smell, i.e. habituate/dishabituate to olfactory stimuli including social odors, also discloses any perturbances in this mechanism leading to impairments in social memory (Silverman et al., 2011). Social behavior has been assessed also in *Shank2* and *Shank3* mouse models. While moderate abnormalities in social interaction were found in *Shank2* mutants (Schmeisser et al., 2012; Won

et al., 2012), a stronger phenotype was evident in most of the *Shank3* models (Peça et al., 2011; Wang et al., 2011; Kouser et al., 2013; Mei et al., 2016; Zhou et al., 2016).

Social behavior has a complex structure in mice. The ability to learn from social experiences and to recognize the conspecifics is a critical factor in maintaining social groups (Barnard et al., 1991; Berry and Bronson, 1992). Mice have a social memory that can last up to several days even after a single encounter with a conspecific. In addition to amygdala, hippocampus appears to be a crucial structure for social memory, integrating complex stimuli required for recognition processes. Long-term social memory further depends on protein synthesis and signaling cascades to be activated (Kogan et al., 2000). Furthermore, in the hippocampus, oxytocin and vasopressin seem to be important regulators of social memory (van Wimersma Greidanus and Maigret, 1996), and mice lacking oxytocin and vasopressin receptor were shown to manifest social memory deficits (Ferguson et al., 2000; Stevenson and Caldwell, 2012). Interestingly, lack of oxytocin was found to massively increase neural activation in hippocampus, suggesting an alternative and compensatory pathway in the hippocampus for the deficits in amygdalar system in the absence of oxytocin (Ferguson et al., 2001). Moreover, in a recent study, oxytocin was shown to boost SHANK1 expression levels *in vitro*, conceivably indicating neuropeptide signaling to influence regulation of scaffolding proteins (Zatkova et al., 2017). Given the abundant expression of SHANK1 protein in hippocampus, SHANK1 deficiency specifically in this structure may be linked to the impairments in social recognition in Study I. However, it remains to be investigated (i) how development plays a role, as juvenile mice with *Shank1* deletion seem to have an intact social memory, and (ii) whether neuropeptide signaling is further involved in this process. As a first step in understanding the role of development, in a preliminary study sociability of adult *Shank1*$^{-/-}$ females was tested by means of the resident-intruder test, where the subject mouse interacts with/habituated to an unfamiliar mouse for four trials, and then subjected to a novel conspecific which allows dishabituation. Increased level of interaction with the novel conspecific implies an intact social recognition, which is impaired in some mouse models (Ferguson et al., 2000). *Shank1*$^{-/-}$ females demonstrated normal sociability, increasing their interaction time when confronted with the novel conspecific. Only significant differences were observed in the first trial, when *Shank1*$^{+/-}$ and *Shank1*$^{-/-}$ mice were interacting with an unfamiliar mouse for the first time, displaying lower levels of social interaction but increasing with repeated trials (unpublished data). Overall, sociability seems to be unaffected when assessed by means of resident-intruder test. However, test duration in this task, which is undoubtedly lasting shorter than that of social

recognition paradigm in three-chambered box assay, might be rather linked to a short-term memory; therefore the processes involved might differ between the two tests, leading to incongruities.

As for communication, findings from Study II are in line with previous reports focusing on *Shank1* model. In the study by Wöhr et al. (2011), communication deficits were evident in mice carrying *Shank1* deletion, not only as pups, but also as adults, shown by reduced scent-marking behavior and inability to adjust USV by males in response to female urine (Wöhr et al., 2011). Concluding the findings from Wöhr et al. (2011) and Study II, it appears that *Shank1* deletion in mice leads to lifelong communication deficits. In terms of comparison to other *Shank* models, however, there are certain discrepancies, but also very similar patterns. For instance, Ey et al. (2013) as well tested *Shank2*$^{-/-}$ mice on a developmental scale and reported altered USV, together with a shift in call emission pattern *Shank2*$^{-/-}$ mice resembling the developmental phenotype in *Shank1*$^{-/-}$ mice. On the other hand, Schmeisser et al. (2012) analyzed USV emission also on a developmental scale, and found that only in the female test group, *Shank2*$^{-/-}$ pups emit more USV than *Shank2*$^{+/+}$ mice on certain PNDs, with no obvious shift in call rate development. Studies employing *Shank3* mouse models reported controversial results in terms of ultrasonic communication. Owing to various mutations found in individuals with ASD, as well as availability of several isoforms of the murine gene, studying *SHANK3* mutations in mice has been under extensive focus, hence several *Shank3* mouse models were created, some of which mimic the mutations in humans granting a higher construct validity (Monteiro and Feng, 2017). In pups, Jaramillo et al. (2016) reported increased number of USV on PND4 and PND6 in one *Shank3* knockout model. In another *Shank3* model, Wang et al. (2016) showed only altered spectral properties of USV on PND4. As of ultrasonic communication in adults, analysis by Bozdagi et al. (2010) revealed fewer USV emitted by *Shank3* mutant males during interaction with females, whereas Wang et al. (2011) on the contrary reported increased number of USV in the presence of females assessed in a different *Shank3* mouse model. Overall, USV emission appears to be mildly affected, and rather controversial if at all, in *Shank3* models for ASD, depicting a complex behavioral phenotype assessed by ultrasonic communication. However, it is worth mentioning that all these models differ in terms of the gene domain and the background mouse strain the mutation was targeted at, which might explain the discrepancies in different studies.

In terms of repetitive behavior, findings from Study III oppose the knowledge on repetitive behaviors, where no changes in self-grooming behavior between genotypes were previously reported in *Shank1* knockout mice (Silverman et al., 2011). Similar results to Study III were however evident in *Shank3* mouse model, with levels of self-grooming being elevated while fewer marbles were buried by *Shank3* mutants (Kouser et al., 2013). In line with these results, elevated levels of self-grooming were also assessed in *Shank2* models (Schmeisser et al., 2012; Won et al., 2012). Nonetheless, a developmental approach in terms of repetitive behavior remains to be further applied in *Shank2* and *Shank3* mouse models for ASD.

4.2 Social Context Plays a Crucial Role in Eliciting ASD-Like Behavioral Phenotype in Shank1 Knockout Mice

Deficits in ASD are observed across multiple contexts (American Psychiatric Association, 2013). Given the reports that individuals with ASD face difficulties in communicating effectively and appropriately in varying everyday social situations (Landry and Loveland, 1989; Helen et al., 2011), a multidisciplinary behavioral investigation across different contexts is suggested in diagnostic assessment of ASD (Lai et al., 2014). In Study II and III, effects of social context on communication and repetitive behavior in *Shank1*$^{-/-}$ mice were investigated by experimentally manipulating the social context. First, in Study II differences in USV production were assessed by adding a social component to the test environment, i.e. odor from the home-cage or a stranger male. Focusing on a single test day where there were no differences in USV emission in the absence of social context, communication deficits in *Shank1*$^{-/-}$ pups became prominent when a social odor was present, regardless of the familiarity of this odor.

The effects of social context on ASD-like behavioral phenotype were further investigated in Study III. With the aim of assessing repetitive behaviors, *Shank1* mice were tested on three consecutive days by means of marble burying test in distinct social contexts, namely in presence or absence of social odors. Evidence for significant differences in self-grooming behavior was obtained between adult *Shank1*$^{-/-}$, *Shank1*$^{+/-}$, and *Shank1*$^{+/+}$ mice. Interestingly, genotype differences were once again most prominent in the social context. Digging behavior was not affected by genotype or social context, whereas marble burying was strongly reduced in adult

Shank1$^{-/-}$ and *Shank1*$^{+/-}$ mice, as compared to *Shank1*$^{+/+}$ controls. Regardless of the social context, *Shank1*$^{-/-}$ and *Shank1*$^{+/-}$ mice buried fewer marbles. Together, findings from the two studies indicate that ASD-related phenotype might be prominently revealed when mice are tested in social contexts. Collectively, these results demonstrate for the first time the critical role of test environment in eliciting ASD-like phenotype in a *Shank* mouse model for ASD.

4.3 Cognitive Deficits in Shank1 Knockout Mice

ID is the most frequently observed comorbid condition in ASD (Matson and Shoemaker, 2009). *Shank1*$^{-/-}$ mice were previously shown to have an aberrant cognitive phenotype, with better performance in some tasks, but impairments in others (Hung et al., 2008). For instance, when *Shank1*$^{-/-}$ mice were tested in the radial maze task as a measure of reference memory performance, they learned faster and more effectively. However, when the long-term stability of spatial memory was assessed after 4 weeks, *Shank1*$^{-/-}$ mice showed a deterioration in their performance and were not able to retain the previous enhancement over *Shank1*$^{+/+}$ mice. The re-learning ability of *Shank1*$^{-/-}$ mice was also better than that of *Shank1*$^{+/+}$ mice, meanwhile when using a more intensive test protocol, both genotypes performed similarly. Besides, after 4 week break from training, *Shank1*$^{-/-}$ mice made more reference memory errors compared to *Shank1*$^{+/+}$ mice. Moreover, contextual fear learning was impaired in *Shank1*$^{-/-}$ mice, while cued fear conditioning was not affected (Hung et al., 2008). Overall, *Shank1*$^{-/-}$ mice seem to display a mixed cognitive phenotype, hence additional evaluation of their cognitive abilities can help unmasking the discrepancies between different tasks. Furthermore, effects of development on cognitive phenotype remain to be assessed.

In Study I, cognitive abilities of juvenile and adult *Shank1*$^{-/-}$ mice were tested by means of novel object recognition task. As juveniles, *Shank1*$^{-/-}$ mice displayed strong impairments in novel object recognition. Object recognition deficits were also evident in adult *Shank1*$^{-/-}$ males, but not in females. Unlike in social recognition paradigm, the auditory cues are missing and olfactory cues are rather limited in the novel object recognition task. Thus, a proper object recognition relies on visual and tactile cues of the stimuli, and inadequate object exploration might lead to

observation of deficits in the object recognition task. However, in a separate experiment in Study I, *Shank1*$^{-/-}$ mice were able to discriminate objects, assessed by object discrimination task. Therefore, the impairments in the novel object recognition task are likely due to general object memory deficits.

Novel object recognition task, together with underlying neuronal processes and brain structures involved, has been extensively studied in rodents, and emerged as a useful tool to assess short-term, intermediate-term, and long-term memory. A network of structures, including hippocampus, as well as perirhinal cortex, having direct and indirect connections to hippocampus, are viewed as fundamental regulatory structures of a proper memory formation (Baxter, 2010). In the hippocampus, specifically in the CA1 region, NMDA receptor activity has been implicated as a critical component for nonspatial memory, including novel object recognition (Rampon et al., 2000). Furthermore, AMPA receptors play a significant role in encoding, retrieval, and consolidation of object memory (Winters and Bussey, 2005). Based on findings from *in vitro* studies, Hung et al. (2008) had postulated that *Shank1* deletion might perturb hippocampus-dependent memory mechanisms due to changes in spine morphology in *Shank1*$^{-/-}$ mice, given the proposal that small spines are rather dynamic and are preferentially involved in acquisition of memory, whereas larger spines are stable and hence constitute the basis of long-term memory storage (Kasai et al., 2003). Without changes in the proportion of AMPA and NMDA receptors, reduced AMPA receptor-mediated synaptic transmission was also demonstrated in *Shank1*$^{-/-}$ mice (Hung et al., 2008). Therefore, deficits in novel object recognition might consequently be originating from altered spine morphology and reduced synaptic transmission in *Shank1*$^{-/-}$ mice.

4.4 Neurobiological Alterations in Shank1 Knockout Mice

In the search for further hints on *Shank1*-hippocampus-memory link, hippocampal brain-derived neurotrophic factor (BDNF) protein expression was assessed after novel object recognition in Study I. BDNF, being abundantly expressed in the hippocampus, cortex, and cerebellum, is involved in modulation of hippocampal long-term potentiation (Korte et al., 1995)

and in processes that require synaptic changes, such as learning and memory (Cowansage et al., 2010). Over the years, several lines of studies suggested that ASD is associated with increased levels of BDNF (Nelson et al., 2001; Miyazaki et al., 2004; Connolly et al., 2006; Correia et al., 2010), in line with the idea that disturbances in activity-dependent signaling pathways are linked to etiology of ASD (Ebert and Greenberg, 2013). Results from Study I showed that hippocampal BDNF protein expression is higher in *Shank1*$^{-/-}$ mice, as compared to *Shank1*$^{+/+}$ littermate controls after novel object recognition paradigm, without changes in basal levels. For a deeper understanding of the expression mechanism, epigenetic regulation of *Bdnf* promoter was further measured; enrichment of H3 acetylation at the *Bdnf* promoter1 was detected. While various studies have previously investigated BDNF levels in mouse models for ASD (Louhivuori et al., 2011; Scattoni et al., 2013; Almeida et al., 2014), and object recognition memory was assessed in *Shank2* (Schmeisser et al., 2012) and *Shank3* mutants (Wang et al., 2011; Yang et al., 2012), findings from Study I are the first linking BDNF expression and its epigenetic regulation with memory deficits in a *Shank* mouse model for ASD. While the direct interaction mechanism between SHANK1 and BDNF is not clearly known, there is evidence for BDNF and postsynaptic scaffold crosstalk. For instance, Catarino et al. (2013) showed that in cultured rat hippocampal neurons, BDNF promotes acetylation of cortactin, suggesting that BDNF may regulate excitatory synapses and PSD-95 dendritic clustering, with cortactin and PSD-95 being direct binding partners of SHANK1 (Naisbitt et al., 1999). It is therefore tempting to speculate that BDNF and SHANK1 might as well regulate each other in the excitatory synapses, with detailed mechanisms demanding further investigation. Catarino et al. (2013) further sought role of epigenetic modifications, and demonstrated that hippocampal neurons treated with trichostatin A, a histone deacetylase inhibitor promoting acetylation, increases SHANK1 and PSD-95 levels. Therefore, this data, together with findings from Study I, adds to the growing evidence that epigenetic modifications are critical in the complex *Shank*-ASD network (Uchino and Waga, 2013; Jiang and Ehlers, 2013).

Primarily focusing on hippocampus, a number of studies have previously provided insights into neurobiological mechanisms in *Shank1* knockout model for ASD (Hung et al., 2008; Mao et al., 2015; Filice et al., 2016). At first glance, Hung et al. (2008) observed a reduction of ~40% in pan-SHANK levels in crude membrane extracts and PSD preparations from *Shank1*$^{-/-}$ mutants, presumably not compensated by SHANK2 or SHANK3. However, protein analysis specific for SHANK2 and/or SHANK3 levels to conclude on a compensatory mechanism has not been

studied in detail yet. In PSDs purified from *Shank1*$^{-/-}$ brains, protein levels of SAPAP (GKAP) and HOMER, two scaffolding proteins that directly bind to SHANK, were found to be reduced. In line with these biochemical results, SAPAP puncta density was reduced and HOMER puncta was more diffused in SHANK1-deficient hippocampal neurons (Hung et al., 2008), supporting the notion that SHANK1 is important for recruiting/stabilizing SAPAP and HOMER at the postsynapse (Tu et al., 1999; Sala et al., 2001). Lack of SHANK1 also altered spine morphology, where mean spine density showed a decrease in the apical dendrites of CA1 pyramidal neurons of hippocampus. SHANK1 deficiency further led to decreased basal synaptic transmission, without affecting synaptic plasticity at Schaffer collateral/CA1 synapses, and the number of AMPA and NMDA receptors. The decreased basal synaptic transmission is presumably caused by the reduced number of functional synapses. These results suggested for the first time *in vivo* that SHANK1 is an important determinant of synaptic strength and morphology at the excitatory synapses (Hung et al., 2008).

Role of SHANK1 has further been investigated in GABAergic, inhibitory, interneurons by focusing on Parvalbumin (PV)-expressing (PV+) fast spiking interneurons in *Shank1*$^{-/-}$ mutants (Mao et al., 2015; Filice et al., 2016). Maintaining excitation/inhibition balance during critical periods of neurodevelopment is crucial, with abnormalities in this circuit being suggested as a pathophysiological hallmark of ASD (Ebert and Greenberg, 2013). PV, acting as a Ca^{2+} buffer, is involved in maintaining the excitation/inhibition balance in the brain. Reductions in PV were previously associated not only with enhanced inhibition at the neuronal level, but also with cognitive deficits including reversal learning impairments in the Morris water maze and T-maze tasks (Wöhr et al., 2015). Mao et al. (2015) showed that, in the hippocampus, SHANK1 is highly expressed in PV+ inhibitory interneurons, and its lack in hippocampal CA1 PV+ interneurons leads to reduced excitatory synaptic inputs and inhibitory synaptic outputs to pyramidal neurons. Further, Mao et al. (2015) addressed the question whether abnormal PV-mediated inhibitory output in *Shank1*$^{-/-}$ mice results in an excitation/inhibition imbalance, and found that, in *Shank1*$^{-/-}$ mice, excitation/inhibition ratio is higher as compared to *Shank1*$^{+/+}$ controls. Given the previous report on reduced excitatory transmission in *Shank1*$^{-/-}$ hippocampal CA1 pyramidal neurons (Hung et al., 2008), the higher excitation/inhibition ratio found by Mao et al. (2015) is thought to be originating from an even more severe decrease in inhibitory transmission in *Shank1*$^{-/-}$ mutants. While reduction of PV was reported in various ASD models (Gogolla et al., 2009) it is important to unveil whether this reduction results from a general neuron loss or PV downregulation. To this

end, Filice et al. (2016) addressed this open question in *Shank1* mouse model for ASD, by focusing on regions expressing high levels of SHANK1. Consequently, they found that the reduction observed in PV+ neurons is due to a reduction in PV mRNA and PV protein levels *per se*, but not due to neuronal cell decrease or loss of PV+ neurons. In contrast, in the striatum, where SHANK1 expression levels are lower, no such differences were observed between *Shank1*$^{-/-}$ mice and *Shank1*$^{+/+}$ controls. Overall, findings from these studies indicate that SHANK1 is an important determinant of synaptic strength and morphology at the excitatory synapses, involved in maintaining the excitation/inhibition balance, and interconnected with Ca^{2+} homeostosome controlling synaptic output (Hung et al., 2008; Mao et al., 2015; Filice et al., 2016).

4.5 Phenotype of Shank1 Knockout Mice - Summary

Concluding the findings from the previous reports and the studies presented here, it appears clear that deletion of *Shank1* gives rise to a mosaic ASD phenotype in mice in relation to diagnostic criteria. In brief, social behavior in *Shank1* knockout mice is moderately affected. Particularly at the early stage of development, communication deficits are evident; with these becoming more prominent when switched to a social context. Likewise, repetitive behaviors are elevated with the presence of a social odor in the test environment. Cognitive performance is impaired throughout development, evidenced by multiple learning experiments. At the neurobiological level, various proteins and mechanisms are affected via deletion of *Shank1* at the synaptic network, in agreement with the idea that ASD pathogenesis extend to epigenetic levels. Remarkably, ASD-like phenotype in *Shank1* mutants appear in both sexes, not following the common male bias.

4.6 Shank1 Mouse Model for ASD: A Translational Perspective

Clinical diagnosis of ASD relies on standardized diagnostic tools which can detect impairments in behavior. In that aspect, behavioral phenotyping of mouse models for ASD can

tell us a lot about the basis of the ASD-relevant deficits. Through three studies, this dissertation aimed to provide a bridge between the two species, carefully considering the relation to human symptoms. Based on findings from these studies, certain analogous deficits could be observed in terms of social interaction, communication, and repetitive behavior, as well as comorbid ID.

In humans, microdeletions in *SHANK1* were reported by Sato et al. (2012), where impairments were evident not only in social communication, but also in repetitive behavior and restricted interests at the early developmental stage in the affected males. For instance, one of the carriers, proband III-5, was engaged in repetitive play and speech when he was 12-24 months old, as reported by his parents. He started speaking in single words at 24 months of age and used phrases by 36 months of age. He has persistently lacked social smiling, facial affect, joint attention, and empathy and his eye contact has always been poor. Another carrier in the family was proband IV-1, who also had signs of ASD phenotype at an early stage of development. His parents detected developmental differences when he was 3 years old, as he lacked interest in other children, but rather was preoccupied with objects, and had an encyclopedic knowledge of cars. He exhibited social deficits, such as having difficulties in eye contact and understanding social cues and rules. He would become upset with changes in routines. Similarly, proband IV-3 showed signs of delayed language acquisition, developing first single words by 24 months of age and mainly communicating led by his parents by the hand. The unrelated male carrier from Sweden also displayed a delay in language acquisition as he started to talk at 2.5 years of age. His speech was formal and pedantic, with an abnormal prosody. He lacked interest in other children, and displayed stereotypic movements when upset.

While in the study by Sato et al. (2012) the affected carriers of the microdeletion were males, females also seem to be affected to a weaker extent. Clinical details on the two females carrying the microdeletion reveal an anxious and shy phenotype in one, and social anxiety disorder in the other. However, they were never diagnosed with ASD or related disorders according to previous diagnostic criteria (e.g. Asperger syndrome). Nonetheless, based on the clinical assessment of individuals with ASD, anxiety disorders are known to co-occur in 42-56% of individuals, of which social anxiety disorder is the most common condition (13-29%), with high-functioning individuals being more susceptible (Lai et al., 2014). Further conclusion from Sato et al. (2012) is the notion that SHANK1 deletions are associated with higher functioning in males, with no effects on IQ levels. As opposed to these findings, however, Wang et al. (2016a) recently

reported a *de novo* mutation in a female diagnosed with ASD, also presented with ID. In line with this report, reductions in auditory working memory capacity were also linked to promoter variant rs3810280 of *SHANK1* gene (Lennertz et al., 2012). Together, two studies support the findings in *Shank1* knockout mice in terms of cognitive functions, with these not being restricted to males. It further brings up the question whether mutations in both species linked to ID could depend on the task used to evaluate the cognitive capability, hence could not necessarily be elicited by conventional intelligence measurements. In conclusion, despite moderate social, communication, and repetitive abnormalities, but stronger phenotype on cognitive deficits, murine *Shank1* deletion appears to be strongly comparable to *SHANK1* mutations in humans.

4.7 Future Perspectives

Extending the scope of previous reports, findings from three studies presented here support compelling evidence for ASD-like traits in *Shank1* mouse model for ASD. Although an in-depth and longitudinal behavioral characterization enables a broad understanding of the phenotype and can serve as markers for a valid model, identifying neurobiological mechanisms underlying the behavioral deficits, as well as developing potential therapeutical approaches remain a major goal in the future of research on *Shank1* model. Priority can be given to rescuing the deficits corresponding to diagnostic symptoms, as well as comorbid cognitive impairments. This approach has been successfully applied in *Shank2* and *Shank3* mouse models for ASD, through targeting glutamatergic pathways at the synapses, and thereby improving the behavioral deficits (Won et al., 2012; Vicidomini et al., 2016). Hung et al. (2008) has shown that via deletion of *Shank1*, expression of HOMER protein is reduced, which presumably disrupts the link of SHANK1 to mGluR. Therefore, restoring glutamatergic receptor function through agonists, such as NMDA receptor partial agonist d-cycloserine or the mGluR5-positive allosteric modulator CDPPB, is an appealing approach in the hope for rescuing the deficits in *Shank1* mutants. Furthermore, enrichment has emerged as another successful strategy to rescue memory deficits, such as object recognition, in CA1-specific NMDA receptor knockout mice (Rampon et al., 2000). However, effects of enrichment on linking glutamatergic function with cognitive impairments have not yet been studied in *Shank* mouse models for ASD.

In addition to glutamatergic alterations, lack of *Shank1* was also associated with reductions in PV (Filice et al., 2016), and excitation/inhibition imbalance in hippocampal CA1 PV+ interneurons (Mao et al., 2015). Although further investigation is required to understand how mutations in *Shank1* contributes to PV pathology, reestablishing GABAergic interneuron function through restoring PV levels appear as an alternative clinical approach to treatment (Rapanelli et al., 2017).

As hippocampus prevails as a fundamental structure, possibly leading to memory deficits with altered protein expression and epigenetic modifications in *Shank1* mutants, it is of great interest to primarily investigate and target this structure. Restoring SHANK expression or developing gene therapies for Shankopathies, for example within a narrow developmental window and only in those circuits underlying the deficits, arise as promising goals to be tackled by the neuroscience community (Carbonetto, 2014). Site- and time-specific gene targeting, such as by means of inducible Cre/lox system, could be a useful strategy in the future.

4.8 Concluding Remarks

> *"The biology of mind bridges the sciences - concerned with the natural world - and the humanities - concerned with the meaning of human experience. Insights that come from this new synthesis will not only improve our understanding of psychiatric and neurological disorders, but will also lead to a deeper understanding of ourselves."*
>
> Eric R. Kandel - Speech at the Nobel Banquet, December 10, 2000[§]

Behavioral neuroscience is a fascinating tool that helps us understand consequences of complex genetic background and environmental factors. While ASD remains a puzzle to be solved, this dissertation aimed to take part in elaborating the single pieces, by focusing on the postsynaptic protein SHANK1. Understanding the function and contribution of a single protein among the profound set of ASD-related proteins remains a challenging task. The efforts put into

[§] "Eric R. Kandel - Banquet Speech". *Nobelprize.org.* Nobel Media AB 2014. Web. 1 May 2017. <http://www.nobelprize.org/nobel_prizes/medicine/laureates/2000/kandel-speech.html>

multiple experiments, however, provide clues to intricate architecture of ASD, with a vast majority of the neurobiological mechanisms seeking answers to the disease pathology. Mice with *Shank1* deletions provide a unique model of the behavioral symptoms characterizing the broad nature of ASD. Overall, findings presented in this dissertation offer the feasibility of an in-depth behavioral phenotyping throughout development and social context in ASD research. Abnormalities in this model that can serve as markers, despite various limitations and challenges, hope to grant basis for future research on mouse models for neurodevelopmental disorders, with the ultimate goal being the treatment of these.

5 PUBLICATIONS

Study I: Aberrant cognitive phenotypes and altered hippocampal BDNF expression related to epigenetic modifications in mice lacking the post-synaptic scaffolding protein SHANK1: Implications for autism spectrum disorder

Received: 13 February 2017 | Revised: 5 April 2017 | Accepted: 3 May 2017

DOI: 10.1002/hipo.22741

RESEARCH ARTICLE

WILEY

Aberrant cognitive phenotypes and altered hippocampal BDNF expression related to epigenetic modifications in mice lacking the post-synaptic scaffolding protein SHANK1: Implications for autism spectrum disorder

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: WO 1732/1-1, Faculty of Psychology, Philipps-University of Marburg, Grant/Award Number: PUM; WO 2012/1.

Abstract

Autism spectrum disorder (ASD) is a class of neurodevelopmental disorders characterized by persistent deficits in social communication/interaction, together with restricted/repetitive patterns of behavior. ASD is among the most heritable neuropsychiatric conditions, and while available evidence points to a complex set of genetic factors, the *SHANK* gene family has emerged as one of the most promising candidates. Here, we assessed ASD-related phenotypes with particular emphasis on social behavior and cognition in *Shank1* mouse mutants in comparison to heterozygous and wildtype littermate controls across development in both sexes. While social approach behavior was evident in all experimental conditions and social recognition was only mildly affected by genotype, *Shank1*$^{-/-}$ null mutant mice were severely impaired in object recognition memory. This effect was particularly prominent in juveniles, not due to impairments in object discrimination, and replicated in independent mouse cohorts. At the neurobiological level, object recognition deficits were paralleled by increased brain-derived neurotrophic factor (BDNF) protein expression in the hippocampus of *Shank1*$^{-/-}$ mice; yet BDNF levels did not differ under baseline conditions. We therefore investigated changes in the epigenetic regulation of hippocampal BDNF expression and detected an enrichment of histone H3 acetylation at the *Bdnf* promoter1 in *Shank1*$^{-/-}$ mice, consistent with increased learning-associated BDNF. Together, our findings indicate that *Shank1* deletions lead to an aberrant cognitive phenotype characterized by severe impairments in object recognition memory and increased hippocampal BDNF levels, possibly due to epigenetic modifications. This result supports the link between ASD and intellectual disability, and suggests epigenetic regulation as a potential therapeutic target.

KEYWORDS
social approach, social recognition, novel object recognition, hippocampus, acetylation

1 | INTRODUCTION

In recent years, deletions, duplications, and coding mutations in the three SH3 and multiple ankyrin repeat domains (SHANK) genes (*SHANK1*, *SHANK2*, and *SHANK3*) have been identified in individuals with autism spectrum disorder (ASD; Bourgeron, 2015; de la Torre-Ubieta, Won, Stein, and Geschwind, 2016; Guilmatre, Huguet, Delorme, and Bourgeron, 2014). First, Durand et al. (2007) described mutations in *SHANK3*, and since then several studies reported mutations in *SHANK1* (Sato et al., 2012), *SHANK2* (Berkel et al., 2010; Leblond et al., 2012; Pinto et al., 2010), and *SHANK3* (Gauthier et al., 2009; Moessner et al., 2007) in ASD and schizophrenia patients with ASD traits. Importantly, *SHANK* mutations were detected in the whole ASD spectrum, with a gradient of severity in intellectual disability (Leblond et al., 2014).

The three *SHANK* genes code for several mRNA splice variants and generate multiple protein isoforms, which assemble into large

molecular platforms at the postsynaptic density (PSD) of excitatory glutamatergic synapses. As master scaffolding proteins, linking glutamate receptors to the actin cytoskeleton and postsynaptic signaling pathways, SHANKs are strongly involved in several synaptic functions, including spine morphogenesis, synapse formation, glutamate receptor trafficking, and activity-dependent neuronal signaling (Sala, Vicidomini, Bigi, Mossa, & Verpelli, 2015; Ting, Peça, & Feng, 2012). In mice, *Shank1* and *Shank2* mRNA expression is particularly high in cortex, hippocampus, and cerebellar Purkinje cells, whereas *Shank3* is most prominently expressed in thalamus, striatum, and granule cells in the cerebellum (Peça et al., 2011). On a subcellular level, SHANK1 proteins are distributed in the body of spines under basal conditions, while SHANK2 and SHANK3 proteins are mostly concentrated in the PSD. In response to depolarization, however, there is a prominent increase in SHANK1 on the tip of the spines, suggesting that SHANK1 is primarily involved in regulating activity-dependent plastic changes of spines (Jiang & Ehlers, 2013; Santini & Klann, 2014; Schmeisser & Verpelli, 2016; Yoo, Bakes, Bradley, Collingridge, & Kaang, 2013).

Hung et al. (2008) generated the first *Shank* mouse model for ASD via disruption of the *Shank1* gene through deletion of exons 14 and 15, leading to a complete knockout of all SHANK1 protein isoforms. Behavioral studies employing *Shank1* mutants provided evidence for an ASD-related phenotype characterized by social communication deficits, alterations in repetitive patterns of behavior, and impairments in cognitive functioning. Specifically, *Shank1* mutants display reduced psychomotor activation and elevated anxiety levels, together with impaired contextual but intact cued fear memory, and enhanced acquisition but impaired retention of spatial memory, with the latter possibly resembling the aberrant cognitive phenotype present in some ASD cases (Hung et al., 2008; Silverman et al., 2011). Consistent with an ASD-relevant behavioral phenotype, *Shank1* mutants further show vocal communication deficts and elevated self-grooming behavior, particularly in social contexts (Sungur, Vörckel, Schwarting, & Wöhr, 2014; 2016; Wöhr, 2014; Wöhr, Roullet, Hung, Sheng, and Crawley, 2011). Finally, Wöhr et al. (2011) observed reduced scent-marking behavior and lack of social modulation of ultrasonic vocalizations in response to female urine in adult male *Shank1* mutants, likely reflecting a failure to learn from social experiences. However, socio-cognitive processes and potential underlying mechanisms have not yet been assessed systematically in *Shank1* mutant mice.

The aims of the current study were therefore twofold. Firstly, at the behavioral level, we aimed to assess ASD-related phenotypes with particular emphasis on social behavior and cognition in *Shank1* mouse mutants in comparison to heterozygous and wildtype littermate controls across development in both sexes. To this aim, juvenile and adult subject mice were tested in two social behavior assays, one for assessing social motivation deficits, i.e. Social Approach, and one for assessing social cognition deficits, i.e. Social Recognition. In addition, a non-social memory task, i.e. Novel Object Recognition, was conducted. Secondly, at the neurobiological level, we analyzed protein expression levels of the brain-derived neurotrophic factor (BDNF), together with its epigenetic regulation, in order to test whether alterations in BDNF expression may contribute to ASD-related behavioral phenotypes displayed by *Shank1* mutants. Dysregulation of activity-dependent signaling pathways in neurons was suggested to play a key role in ASD etiology (Ebert & Greenberg, 2013) and several lines of evidence indicate that ASD is associated with increased BDNF expression (Bourgeron, 2015; Nickl-Jockschat & Michel, 2011). BDNF is a small dimeric protein whose expression is abundant in the hippocampus, cortex, and cerebellum. It plays a diverse role in regulating neuronal structure and function during development and in the adult nervous system, modulating synaptic plasticity and thus learning and memory (Cowansage, LeDoux, & Monfils, 2010; Park & Poo, 2013).

2 | MATERIALS AND METHODS

2.1 | Animals and housing

Juvenile and adult *Shank1*$^{-/-}$ null mutant mice with a targeted replacement of exons 14 and 15 encoding almost the entire PDZ domain were compared to *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{+/+}$ wildtype littermate control mice. Mice were obtained from mutant lines originally generated by Hung et al. (2008) through injection of targeted embryonic stem cell clones into C57BL/6J blastocysts and backcrossing of *Shank1*$^{+/-}$ offspring into C57BL/6J and 129SvJae strains. As high mortality rates were seen in the C57BL/6J background strain and very low locomotion in the 129SvJae background strain (Hung et al., 2008; Silverman et al., 2011), the two lines were crossed for at least three generations to produce a mixed C57BL/6J/129SvJae background for the *Shank1* mutation. This mixed background was maintained and used in the present study, consistent with other studies focusing on this *Shank1* mutant (Hung et al., 2008; Silverman et al., 2011; Sungur et al., 2014, 2016; Wöhr, 2014; Wöhr et al., 2011). Using a heterozygous breeding protocol, *Shank1*$^{+/-}$ males and females were bred in a conventional vivarium at the Biomedical Research Center of the Philipps-University of Marburg, Germany. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. After weaning on PND21, mice were socially housed in groups of 2–6 with same-sex partners in polycarbonate Makrolon type III IVC cages (LxWxH: 420 × 265 × 180 mm, 825 cm^2; Ehret, Emmendingen, Germany). Bedding and a wooden board were provided in each cage. Standard rodent chow and water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle (lights on: 06:00 h) at approximately 22°C and 40–50% humidity. Pups were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum Manufacturing Inc., Brockville, ON, Canada). The ink was inserted subcutaneously through a 30 gauge hypodermic needle tip into the center of the paw. For genotyping, mouse tail snips were collected by dissecting ~0.3 cm of tail between PND3–12. Genotyping was performed as described previously (Sungur et al., 2014). All procedures were approved by the ethical committee of the local government (Regierungspräsidium Gießen, Germany).

2.2 | Behavioral procedures – overview

For detecting ASD-related behavioral phenotypes, subject mice of the first cohort were tested in two social behavior assays, one for assessing social motivation deficits, i.e. Social Approach, and one for assessing social cognition deficits, i.e. Social Recognition. In addition, a non-social memory task, i.e. Novel Object Recognition, was conducted. All three behavioral assays were performed in a three-chambered box. Subject mice of both sexes were tested as juveniles between 3 and 6 weeks of age and again as adults between 17 and 21 weeks of age. At both developmental stages, behavioral testing was conducted on three consecutive days. On the first day, subject mice were individually kept for 30 min in a Makrolon type III IVC cage and were then allowed to explore the empty three-chambered box for 30 min in order to habituate to the apparatus. On the second and third day, subject mice were again first individually kept for 30 min. Immediately after the 30 min isolation period, social behavior assays and the non-social memory task were performed in a balanced order, with Social Approach and Social Recognition being performed on one day and Novel Object Recognition the other day. Body weight was measured after behavioral testing. A second independent cohort of juvenile mice was tested in Object Discrimination in order to differentiate between object recognition versus discrimination deficits. Finally, a third cohort of juvenile mice was subjected only to Novel Object Recognition for the assessment of learning-associated BDNF expression levels. Behavioral testing was performed under dim red light during the light phase of the 12:12 light/dark cycle. Prior to each test, behavioral equipment was thoroughly cleaned using a 0.1% acetic acid solution followed by drying. Experimenters were blind to genotypes during data acquisition and analysis.

2.3 | Three-chambered box

Novel Object Recognition, Social Approach and Social Recognition, were conducted in a three-chambered box. The box was made of black polycarbonate material and consisted of two side chambers (230 × 345 × 350 mm) connected through a smaller chamber (145 × 70 × 350 mm) located centrally between the two side chambers. This middle chamber had two retractable doors to control access to the side chambers. Lack of side preference was confirmed during the 30 min habituation on the first day. The three-chambered box was equipped with two UltraSoundGate Condenser Microphones CM16 sensitive to frequencies of 15–180 kHz (flat frequency response between 25 and 140 kHz; ±6 dB; Avisoft Bioacoustics, Berlin, Germany), one in each side chamber. They were connected via an UltraSoundGate 416 USGH audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz (16 bit) by Avisoft RECORDER (version 2.97).

2.4 | Novel object recognition

After being individually kept for 30 min in a Makrolon type III IVC cage, subject mice were tested for Novel Object Recognition, using a modified protocol previously established (Bevins & Besheer, 2006; Valluy et al., 2015). This test consisted of three phases, i.e. object acquisition trial (10 min), inter-trial interval (30 min), and object recognition trial (10 min). In the object acquisition trial, each subject mouse was allowed to freely explore for 10 min the three-chambered box containing two identical sample objects, with one sample object being centrally placed in each of the two side chambers. After the object acquisition trial, the subject mouse was individually kept for 30 min in the previously used Makrolon type III IVC cage, the inter-trial interval. During that time, one of the objects from the object acquisition trial (*familiar* object) was replaced with a novel object of similar size but different in color, shape, and material (*novel* object) to test object recognition memory. Specifically, one clean familiar object and one clean novel object were placed into the three-chambered box, where the two identical objects had been located during the object acquisition trial. After the 30 min delay, each subject mouse was returned to the three-chambered box for a 10 min object recognition trial and allowed to freely explore the familiar and the novel object. As objects, two glass cylinders (60 mm in diameter, 80 mm high) and two red metal cubes (50 × 50 × 88 mm) were used. Location and type of objects presented were counter-balanced between subject mice. Genotypes did not show a preference for either of the alike objects during the object acquisition trial (all p-values >0.050).

2.5 | Object discrimination

Object Discrimination was performed to control for the ability of the subject mice to distinguish between the objects used for Novel Object Recognition. To this aim, mice were presented with three *identical* objects and one *unique* object of similar size, but different in color, shape, and material, in the four square parcels of an open field (40 × 40 cm). Type and position of the *unique* object was balanced between subjects. Each mouse was placed into the open field for 10 min and allowed to freely explore it.

2.6 | Social approach and social recognition

After being individually kept for 30 min in a Makrolon type III IVC cage, subject mice were tested for Social Approach and Social Recognition, using a modified protocol previously established (Wöhr et al., 2013). As in the Novel Object Recognition test, testing consisted of three phases, i.e. social approach trial (10 min), inter-trial interval (30 min), and social recognition trial (10 min). In the social approach trial, each subject mouse was allowed to freely explore for 10 min the three-chambered box containing an empty wired-cage (*object*) on one side and a stimulus mouse constrained in an identical wired-cage (*animal*) on the other side. After the social approach trial, the subject mouse was individually kept for 30 min in the previously used Makrolon type III IVC cage, the inter-trial interval. Then, following the 30 min delay, each subject mouse was returned to the three-chambered box for a 10 min social recognition trial. During the social recognition trial, subject mice were given the choice between the stimulus mouse from the previous social approach trial (*familiar* mouse) on the side where it was presented before and a novel stimulus mouse replacing the empty wired-cage

(*novel* mouse) on the other side. As stimulus mice, age- and sex-matched C57BL/6J mice (Charles River Laboratories, NC, USA) were used. Stimulus mice were group-housed under similar conditions as subject mice and habituated to the wired-cages for 30 min prior testing. Location and stimulus mice presented were counter-balanced between subject mice.

2.7 | Behavior analysis

For Novel Object Recognition, Social Approach and Social Recognition, number of entries into the chambers, the time spent therein, and object investigation were scored from videos using Noldus Observer XT software (Noldus Information Technology, Wageningen, The Netherlands) on a personal computer by a trained observer blind to genotypes. Object investigation was defined as time spent sniffing the social stimulus/object when the nose was oriented towards it, with the nose-object distance being 3 cm or less. Novel Object Recognition and Social Recognition were defined as spending significantly more time sniffing the *novel* than the *familiar* object or mouse, respectively.

2.8 | Ultrasonic vocalization analysis

For Social Approach and Social Recognition in juveniles, ultrasonic vocalizations were analyzed with Avisoft-SASLab Pro software (Version 5.2.05; Avisoft Bioacoustics). A fast Fourier transform was conducted (512 FFT length, frame size: 100%, Hamming Window and 75% time-window overlap), producing spectrograms at 488 Hz frequency resolution and 0.512 ms temporal resolution. Ultrasonic vocalizations were marked and counted by a trained observer blind to genotypes. As ultrasonic vocalizations were recorded in both side chambers, they were analyzed separately. Afterwards each call was matched to the side it was recorded at with higher peak amplitude. Total call number was then calculated for each side.

2.9 | Protein analysis

Learning-associated BDNF protein expression in the hippocampus was quantified using enzyme-linked immunosorbent assay (ELISA), with half of the mice being subjected to Novel Object Recognition whereas the other half not being exposed to this learning experience, i.e. home cage controls. Mice were sacrificed directly after Novel Object Recognition and their left and right hippocampi were removed, snap-frozen on dry ice, and stored at −80°C until processed. BDNF protein levels were measured using the Quantikine ELISA Kit (R&D Systems, Wiesbaden, Germany) and total protein levels were measured using Pierce BCA Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. BDNF protein concentrations in the hippocampi were compared between genotypes in relation to total protein levels, with missing concentrations being imputed where necessary.

2.10 | Epigenetic analysis

Epigenetic regulation of hippocampal BDNF expression through histone acetylation was assessed by means of chromatin immunoprecipitation (ChIP; Tessarz & Kouzarides, 2014). Hippocampi were placed in 450 μl RPMI medium (Sigma-Aldrich, Taufkirchen, Germany) plus 50 μl trypsin solution (Capricorn, Ebsdorfergrund, Germany) and incubated for 20 min at 37°C. Afterwards, 500 μl DNase solution (Sigma-Aldrich) was added and incubated for another 5 min at room temperature. The medium was carefully aspirated and tissues were washed twice with PBS (Sigma-Aldrich). Hippocampi were then resuspended in 500 μl RPMI medium, placed in 100 mm bacteriological grade petri dishes and gently dissociated by 8–10 times repeated aspiration through a Pasteur pipette resulting in homogenous cell suspensions which were subsequently washed once with 1 ml RPMI medium. DNA-protein interactions in isolated cells were cross-linked in 1% formaldehyde for 10 min at room temperature. Subsequently, ChIP was performed, as described recently (Harb et al. 2015), using antibodies against acetylated histone H3 (H3ac; Merck Millipore, Darmstadt, Germany) and acetylated histone H4 (H4ac; Merck Millipore). Immunoprecipitated and eluted DNA was purified with QIAquick columns (Qiagen, Hilden, Germany) and amplified by means of quantitative PCR using primers specific for the murine promoters of *Bdnf* (promoter1; *Bdnf* forward: 5′-TTGGTCACGTAACTGGCT-3′; *Bdnf* reverse: 5′-TGGGGAACTTGTTGCTTT-3′), *interleukin (Il)2* (*Il2* forward: 5′-TAAATAAGGCCATAGAATGG-3′; *Il2* reverse: 5′- GTTACATTAGCCCACACTTA-3′), *Il6* (*Il6* forward: 5′-GACATGCCAAAGTGCTGAGTCACT-3′; *Il6* reverse: 5′-AGACTCATGGGAAAATCCCACATT-3′), *tumor necrosis factor (Tnf)*-α promoter (*Tnf*-α forward: 5′-TGGGTTTCAGTTCTCAGGGT-3′; *Tnf*-α reverse: 5′-GGGTTTGGAAAGTTGGGGAC-3′) and the housekeeping gene *Rpl32* promoter (*Rpl32* forward: 5′-TCATTTCTCAGGCACATCTT-3′; *Rpl32* reverse: 5′-ACTCACCGTAAAACAGATGG-3′), respectively. All amplifications were performed in duplicate by using 1 μl of DNA per reaction. For analysis, the percent enrichment of the negative control (IgG) was subtracted from percent enrichment in H3 or H4 ChIP and normalized to the housekeeping gene *Rpl32* results.

2.11 | Statistical analysis

Body weight was analyzed using a univariate ANOVA with the between-subject factor genotype. Novel Object Recognition, Social Approach, and Social Recognition were analyzed using ANOVAs for repeated measurements with the between-subject factor genotype and the within-subject factor social stimulus/object. When appropriate, ANOVAs were followed by Bonferroni post-hoc tests for comparing genotypes or paired t-tests for comparing objects within genotypes. As no sex differences were expected in juveniles, males and females were pooled; whereas in adulthood, males and females were analyzed separately. For Novel Object Recognition and Social Recognition, behavior recorded in the first 5 min of each trial was included in the statistical analysis, since habituation to novel stimuli is likely to occur in testing periods exceeding 5 min (Bevins & Besheer, 2006). BDNF protein levels were analyzed using ANOVAs for repeated measurements with the between-subject factor genotype and the within-subject factor brain hemisphere. Pearson's correlation coefficients were calculated in order to test whether BDNF concentrations from left and right hemispheres covaried, whenever values were available for both hemispheres.

Epigenetic regulation, i.e. H3 and H4 acetylation levels, were compared between genotypes by means of a multivariate ANOVA. Data are presented as means ± standard errors of the mean (SEM).

3 | RESULTS

3.1 | Body weights

In juveniles, genotype-dependent differences in body weight were detected ($F_{2,75}$=3.832, $p = 0.026$). Juvenile *Shank1*$^{-/-}$ mice (11.423 ± 0.589 g) weighed less than *Shank1*$^{+/+}$ littermate controls (13.986 ± 0.681 g; $p = 0.025$). Such genotype-dependent differences were even more prominent in adulthood and evident in males ($F_{2,32}$=8.120, $p = 0.002$) and females ($F_{2,42}$=11.355, $p < 0.001$), with *Shank1*$^{-/-}$ mice weighing less than *Shank1*$^{+/+}$ littermate controls, both in males (28.080 ± 0.504 g vs. 34.118 ± 1.250 g; $p = 0.001$) and females (21.131 ± 0.770 g vs. 27.591 ± 1.420 g; $p = 0.001$). Across development, *Shank1*$^{+/-}$ mice displayed an intermediate phenotype (not shown in detail). The body weight data are consistent with previous studies (Sungur, Schwarting, & Wöhr, 2016; Wöhr et al., 2011).

3.2 | Novel object recognition

3.2.1 | Juvenile mice

Evidence for genotype-dependent differences in object recognition memory was obtained in juvenile mice (genotype: $F_{2,66}$=6.083, $p = 0.004$; object: $F_{1,66}$=14.885, $p < 0.001$; genotype x object: $F_{2,66}$=2.446, $p = 0.094$). Juvenile *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ mice preferred to sniff the *novel* over the *familiar* object after a 30 min delay (T_{16}=2.375, $p = 0.030$ and T_{25}=3.295, $p = 0.003$; respectively), reflecting intact object recognition memory. Juvenile *Shank1*$^{-/-}$ mice, however, showed no preference for sniffing the *novel* over the *familiar* object (T_{25}=0.693, $p = 0.495$; Figure 1), indicating object recognition deficits. Of note, genotypes also differed in exploring objects during the object acquisition trial (genotype: $F_{2,66}$=8.334, $p = 0.001$; object: $F_{1,66}$=1.431, $p = 0.236$; object x genotype: $F_{2,66}$=0.312, $p = 0.733$). Juvenile *Shank1*$^{-/-}$ mice spent less time sniffing objects than *Shank1*$^{+/+}$ littermate controls ($p < 0.001$) and object recognition deficits displayed by *Shank1*$^{-/-}$ mice might thus appear to be due to insufficient object exploration. However, this appears unlikely for two reasons: (1) Juvenile *Shank1*$^{-/-}$ mice spent a similar amount of time sniffing objects during the object acquisition trial as *Shank1*$^{+/-}$ littermate controls ($p = 0.103$), with the latter not displaying object recognition deficits. (2) When focusing on the upper quartile of *Shank1*$^{-/-}$ mice, which spent a similar amount of time exploring the objects during the object acquisition trial as *Shank1*$^{+/+}$ littermate controls (T_{22}=1.299, $p = 0.207$), still no evidence for intact object recognition memory was obtained (T_6=1.153, $p = 0.293$), indicating persistence of object recognition deficits despite adequate object exploration.

3.2.2 | Adult mice – males

Genotype-dependent differences in object recognition memory were further observed in adult males (genotype: $F_{2,27}$=0.347, $p = 0.710$; object: $F_{1,27}$=7.774, $p = 0.010$; genotype x object: $F_{2,27}$=3.762, $p = 0.036$), consistent with the data obtained in juvenile mice. Adult *Shank1*$^{+/+}$ males displayed intact object recognition memory, as reflected in more time spent sniffing the *novel* over the *familiar* object (T_7=2.702, $p = 0.031$). No such preference was seen in adult *Shank1*$^{+/-}$ (T_{11}=0.733, $p = 0.479$) and *Shank1*$^{-/-}$ (T_9=0.338, $p = 0.743$) males, with both genotypes spending a similar amount of time sniffing *novel* and *familiar* objects (Figure 2a), indicating object recognition deficits. Genotypes did not differ in exploring objects during the object acquisition trial (genotype: $F_{2,27}$=0.044, $p = 0.957$; object: $F_{1,27}$=0.930, $p = 0.343$; genotype x object: $F_{2,27}$=1.799, $p = 0.185$).

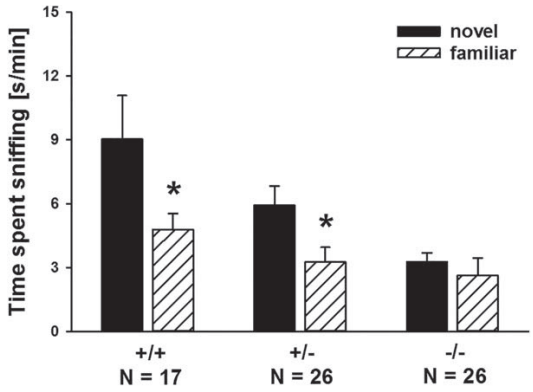

FIGURE 1 Novel Object Recognition in juvenile *Shank1* mice. Novel Object Recognition is depicted by time spent sniffing (seconds per minute) the *novel* object (black bar) over the *familiar* object (striped bar). *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ mice displayed significantly more time sniffing the *novel* than the *familiar* object. In contrast, *Shank1*$^{-/-}$ mice displayed no preference for sniffing the *novel* over the *familiar* object, indicating object memory deficits. Data are presented as means + standard errors of the mean (SEM). *$p < 0.050$ preference vs. *novel* object

3.2.3 | Adult mice – females

Similar to adult males, evidence for genotype-dependent differences in object recognition memory was obtained in adult females (genotype: $F_{2,36}$=5.555, $p = 0.008$; object: $F_{1,36}$=39.318, $p < 0.001$; genotype x object: $F_{2,36}$=2.306, $p = 0.114$). Yet, effects were weaker, with adult females of all genotypes displaying intact object recognition memory (*Shank1*$^{+/+}$: T_7=3.432, $p = 0.011$; *Shank1*$^{+/-}$: T_{14}=3.694, $p = 0.002$; *Shank1*$^{-/-}$: T_{15}=3.231, $p = 0.006$), although the preference for the *novel* over the *familiar* object was clearly less prominent in adult *Shank1*$^{-/-}$ females (Figure 2b). Genotypes also differed in exploring objects during the object acquisition trial (genotype: $F_{2,36}$=4.328, $p = 0.021$; object: $F_{1,36}$=5.716, $p = 0.022$; object x genotype: $F_{2,36}$=0.065, $p = 0.937$). During acquisition, *Shank1*$^{-/-}$ females spent less time exploring objects than *Shank1*$^{+/+}$ ($p = 0.020$) but not *Shank1*$^{+/-}$ ($p = 0.280$).

3.3 | Social approach and social recognition

3.3.1 | Juvenile mice

No evidence for genotype differences in social approach behavior was obtained in juvenile mice (genotype: $F_{2,67}$=0.150, $p = 0.861$; stimulus:

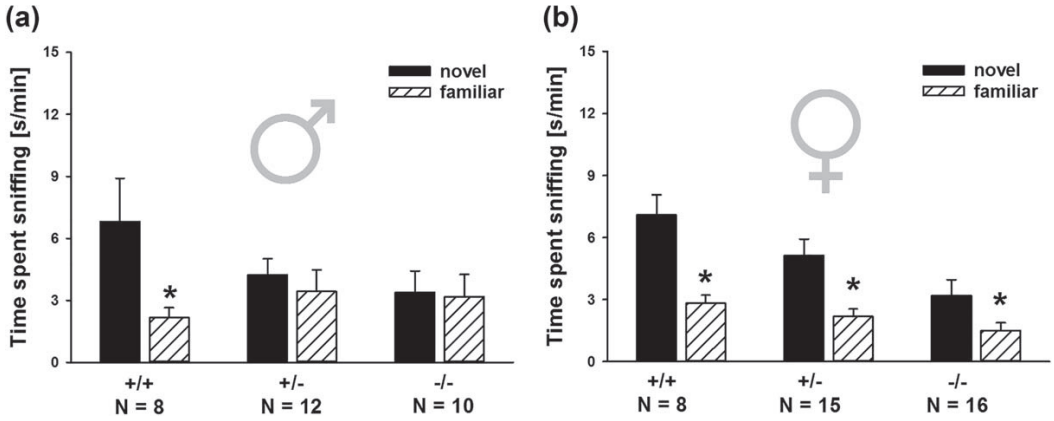

FIGURE 2 Novel Object Recognition in adult *Shank1* mice. Novel Object Recognition is depicted by time spent sniffing (seconds per minute) the *novel* object (black bar) over the *familiar* object (striped bar). (a) Male *Shank1*$^{+/+}$ mice displayed significantly more time sniffing the *novel* than the *familiar* object. In contrast, male *Shank1*$^{+/-}$ and *Shank1*$^{-/-}$ displayed no preference for sniffing the *novel* over the *familiar* object, indicating object memory deficits. (b) All of the female *Shank1* genotypes displayed significantly more time sniffing the *novel* than the *familiar* object. Data are presented as means + SEM. *$p < 0.050$ preference vs. *novel* object

$F_{1,67}$=95.898, $p < 0.001$; genotype x stimulus: $F_{2,67}$=0.258, $p = 0.774$). All genotypes spent significantly more time sniffing the *animal* over the *object*, reflecting intact social motivation. Specifically, significant preferences for the *animal* were seen in juvenile *Shank1*$^{+/+}$ (T_{16}=4.893, $p < 0.001$), *Shank1*$^{+/-}$ (T_{26}=6.772, $p < 0.001$) and *Shank1*$^{-/-}$ (T_{25}=5.869, $p < 0.001$) mice (Figure 3a). Concomitant recordings of ultrasonic vocalizations revealed that mice vocalized more in proximity to the *animal* than the *object*, irrespective of genotype (genotype: $F_{2,67}$=0.077, $p = 0.926$; stimulus: $F_{1,67}$=12.898, $p = 0.001$; genotype x stimulus: $F_{2,67}$=0.252, $p = 0.778$; not shown in detail), mirroring the behavioral findings. Likewise, no evidence for genotype differences in social recognition memory was obtained (genotype: $F_{2,67}$=0.566, $p = 0.571$; stimulus: $F_{1,67}$=46.093, $p < 0.001$; genotype x stimulus: $F_{2,67}$=0.71, $p = 0.932$). All genotypes spent significantly more time sniffing the *novel* over the *familiar* stimulus mouse, indicating that all genotypes were able to discriminate between the *novel* and the *familiar* stimulus mouse after a delay of 30 min and thus reflecting intact social cognition. Specifically, significant preferences for the *novel* stimulus mouse were seen in juvenile *Shank1*$^{+/+}$ (T_{16}=3.132, $p = 0.006$), *Shank1*$^{+/-}$ (T_{26}=5.101, $p < 0.001$) and *Shank1*$^{-/-}$ (T_{25}=3.809, $p = 0.001$) mice (Figure 3b). Social recognition was not reflected in ultrasonic vocalizations, with emission rates not differing between the *novel* and the *familiar* stimulus mouse, irrespective of genotype (genotype: $F_{2,67}$=0.760, $p = 0.472$; stimulus: $F_{1,67}$=0.322, $p = 0.572$; genotype x stimulus: $F_{2,67}$=1.534, $p = 0.223$; not shown in detail).

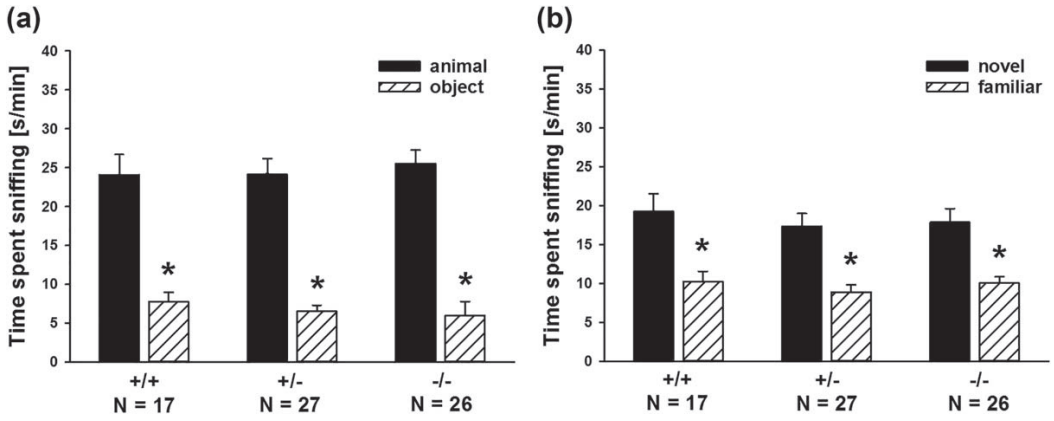

FIGURE 3 Social Approach and Social Recognition in juvenile *Shank1* mice. (a) Social Approach is depicted by time spent sniffing (seconds per minute) the *animal* (black bar) over the *object* (striped bar). All of the *Shank1* genotypes displayed normal social approach behavior, spending significantly more time sniffing the *animal* than the *object*. (b) Social Recognition is depicted by time spent sniffing (seconds per minute) the *novel* animal (black bar) over the *familiar* animal (striped bar). All of the *Shank1* genotypes displayed normal social recognition behavior, spending significantly more time sniffing the *novel* than the *familiar* animal. Data are presented as means + SEM. *$p < 0.050$ preference vs. *animal* and *novel* animal, respectively

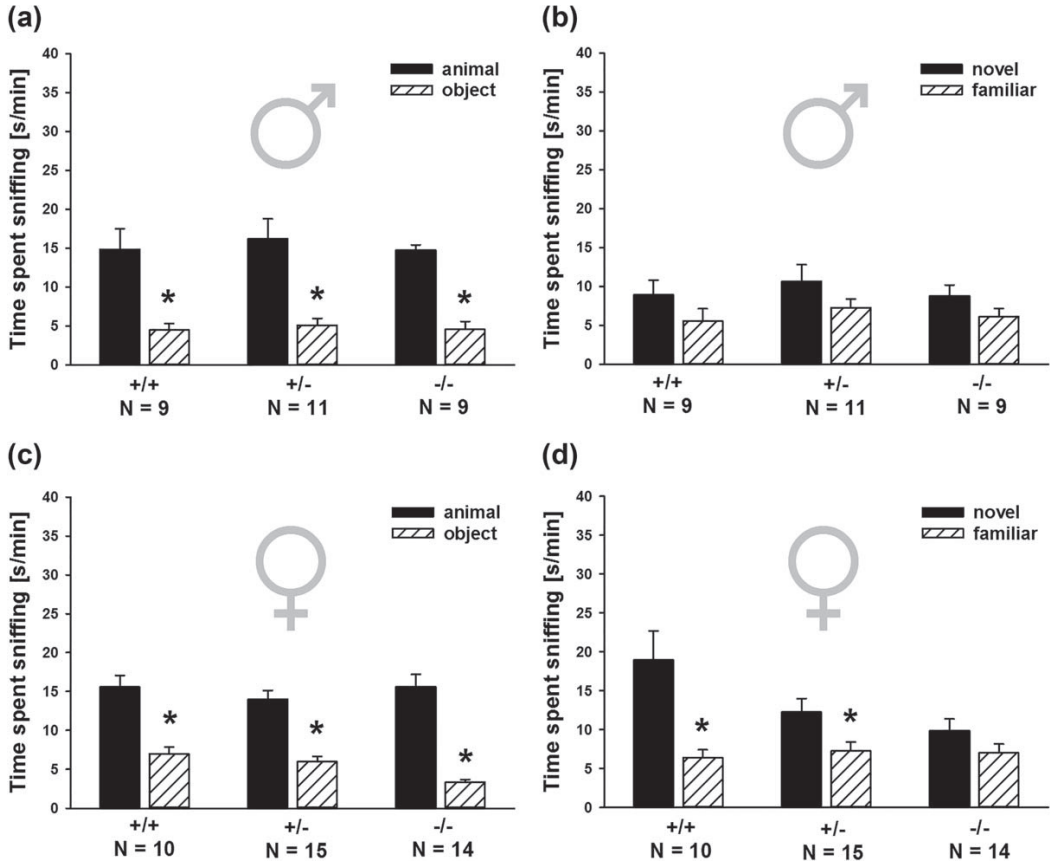

FIGURE 4 Social Approach and Social Recognition in adult *Shank1* mice. (a) Social Approach is depicted by time spent sniffing (seconds per minute) the *animal* (black bar) over the *object* (striped bar). All of the male *Shank1* genotypes displayed normal social approach behavior, spending significantly more time sniffing the *animal* than the *object*. (b) Social Recognition is depicted by time spent sniffing (seconds per minute) the *novel* animal (black bar) over the *familiar* animal (striped bar). None of the male *Shank1* genotypes displayed a preference for sniffing the *novel* over the *familiar* animal, indicating social recognition deficits. (c) All of the female *Shank1* genotypes displayed normal social approach behavior, spending significantly more time sniffing the *animal* than the *object*. (d) Female $Shank1^{+/+}$ and $Shank1^{+/-}$ displayed significantly more time sniffing the *novel* than the *familiar* animal. In contrast, female $Shank1^{-/-}$ mice displayed no preference for sniffing the *novel* over the *familiar* animal, indicating social recognition deficits. Data are presented as means + SEM. $^*p < 0.050$ preference vs. *animal* and *novel* animal, respectively

3.3.2 | Adult mice – males

Consistent with the data obtained in juvenile mice, no evidence for genotype differences in social approach behavior was obtained, with all genotypes spending significantly more time sniffing the *animal* over the *object* (stimulus: $F_{1,26}=73.926$, $p<0.001$; genotype: $F_{2,26}=0.196$, $p=0.824$; genotype x stimulus: $F_{2,26}=0.057$, $p=0.945$), reflecting intact social motivation in adult males. Specifically, significant preferences for the *animal* were seen in male $Shank1^{+/+}$ ($T_8=3.664$, $p=0.006$), $Shank1^{+/-}$ ($T_{10}=5.488$, $p<0.001$), $Shank1^{-/-}$ ($T_8=8.722$, $p<0.001$) mice (Figure 4a). Furthermore, as in juvenile mice, no evidence for genotype differences in social recognition memory was obtained (stimulus: $F_{1,26}=7.569$, $p=0.011$; genotype: $F_{2,26}=0.554$, $p=0.581$; genotype x stimulus: $F_{2,26}=0.042$, $p=0.959$). However, effects were much weaker than in juveniles and thus no significant preferences for the *novel* stimulus mouse were seen at the level of individual genotypes ($Shank1^{+/+}$: $T_8=1.318$, $p=0.224$; $Shank1^{+/-}$: $T_{10}=1.867$, $p=0.091$; $Shank1^{-/-}$: $T_8=1.896$, $p=0.095$; Figure 4b).

3.3.3 | Adult mice – females

As in adult males, no evidence for genotype differences in social approach behavior was obtained, with all genotypes spending significantly more time sniffing the *animal* over the *object* (genotype: $F_{2,36}=1.309$, $p=0.283$; stimulus: $F_{1,36}=104.667$, $p<0.001$; stimulus x genotype: $F_{2,36}=2.141$, $p=0.132$), reflecting intact social motivation in adult females. Specifically, significant preferences for the *animal* were seen in female $Shank1^{+/+}$ ($T_9=4.851$, $p=0.001$), $Shank1^{+/-}$ ($T_{14}=5.513$, $p<0.001$), and $Shank1^{-/-}$ ($T_{13}=7.571$, $p<0.001$) mice (Figure 4c). In contrast to adult males, however, social recognition memory in adult females was affected by genotype (stimulus: $F_{1,36}=19.158$, $p<0.001$; genotype: $F_{2,36}=2.916$, $p=0.067$; genotype

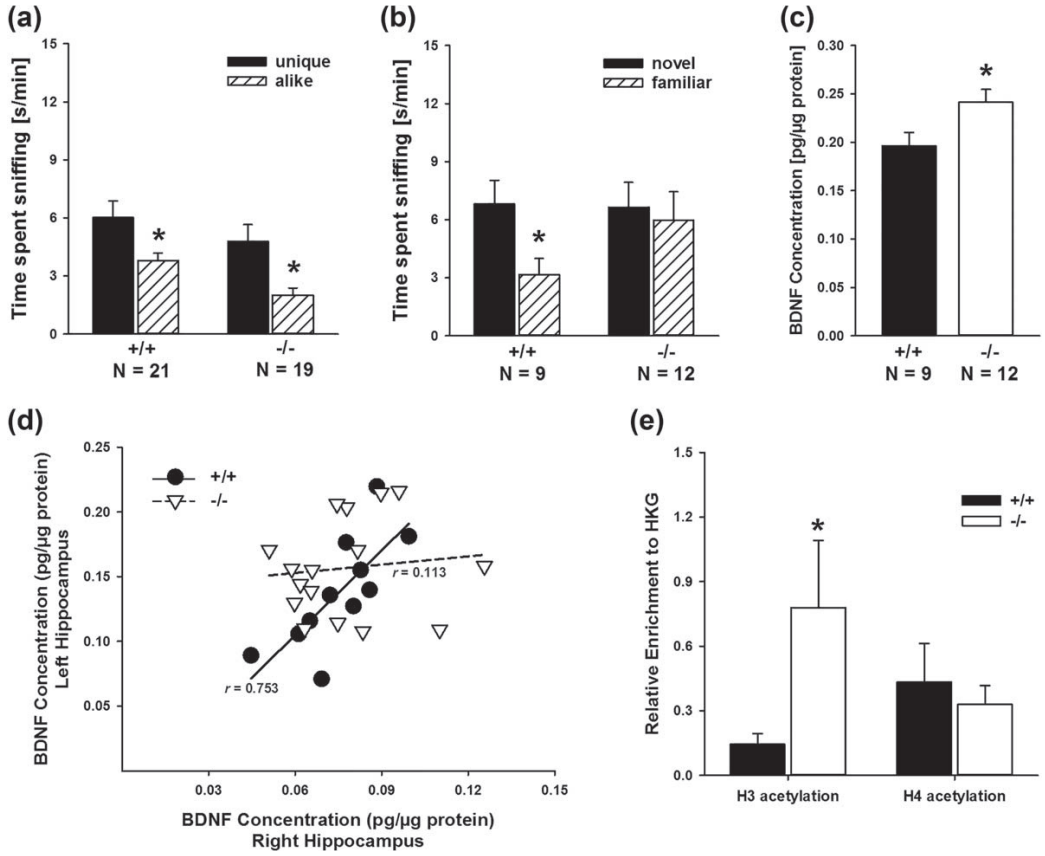

FIGURE 5 Object Discrimination, Novel Object Recognition, and hippocampal brain-derived neurotrophic factor (BDNF) protein and histone acetylation levels in juvenile *Shank1* mice. (a) Object Discrimination is depicted by time spent sniffing (seconds per minute) a *unique* object (black bar) over three *alike* objects (average; striped bar). Both *Shank1*$^{+/+}$ and *Shank1*$^{-/-}$ mice were able to discriminate the *unique* object, spending significantly more time sniffing the *unique* object than the *alike* objects. (b) Novel object recognition is depicted by time spent sniffing (seconds per minute) the *novel* object (black bar) over the *familiar* object (striped bar). In a separate cohort of mice dedicated to hippocampal BDNF measurements, *Shank1*$^{+/+}$ mice displayed significantly more time sniffing the *novel* than the *familiar* object. In contrast, *Shank1*$^{-/-}$ mice displayed no preference for sniffing the *novel* over the *familiar* object, again indicating object memory deficits. (c) Significantly elevated BDNF protein levels were evident in the hippocampi of *Shank1*$^{-/-}$ mice (white bar) in comparison to *Shank1*$^{+/+}$ mice (black bar). (d) Correlation between BDNF protein levels of the left and right hippocampus in *Shank1* mice. BDNF protein concentrations were significantly correlated with one another in *Shank1*$^{+/+}$ mice (black circles) but not in *Shank1*$^{-/-}$ mice (white circles). (e) Histone 3 (H3) and 4 (H4) acetylation levels are depicted by enrichment levels (relative to housekeeping gene; HKG). Significantly elevated H3 but not H4 acetylation levels were revealed in the hippocampi of the *Shank1*$^{-/-}$ mice (white bar) in comparison to *Shank1*$^{+/+}$ mice (black bar). Data are presented as means + SEM. *$p < 0.050$ preference vs. *unique* object, *novel* object, and *Shank1*$^{+/+}$ mice, respectively

x stimulus: $F_{2,36}=3.215$, $p = 0.052$). While adult *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ females spent more time sniffing the *novel* than the *familiar* stimulus mouse ($T_9=2.819$, $p = 0.020$ and $T_{14}=2.665$, $p = 0.019$; respectively), adult *Shank1*$^{-/-}$ females did not discriminate the *novel* stimulus mouse from the *familiar* one ($T_{13}=1.394$, $p = 0.187$; Figure 4d), indicating social recognition deficits in *Shank1*$^{-/-}$ females.

3.4 | Learning-associated hippocampal BDNF protein expression and epigenetic regulation

Object recognition deficits in *Shank1*$^{-/-}$ mice were not due to impairments in discriminatory abilities. Specifically, when exposing an independent cohort of mice to three *identical* and one *unique* object at the same time, juvenile *Shank1*$^{+/+}$ and *Shank1*$^{-/-}$ mice both displayed a preference for the *unique* object ($T_{20}=2.111$, $p = 0.048$ and $T_{18}=2.845$, $p = 0.011$; respectively; Figure 5a). Moreover, object recognition deficits in *Shank1*$^{-/-}$ mice were replicated in a separate cohort of juvenile animals. While juvenile *Shank1*$^{+/+}$ mice preferred to sniff the *novel* over the *familiar* object after a 30 min delay as expected ($T_8=2.548$, $p = 0.034$), juvenile *Shank1*$^{-/-}$ mice again showed no preference for sniffing the *novel* over the *familiar* object ($T_{11}=0.577$, $p = 0.576$; Figure 5b). In the same cohort, alterations in hippocampal BDNF protein levels were determined and genotype-dependent differences detected. Specifically, hippocampal BDNF protein expression was found to be higher in *Shank1*$^{-/-}$ mice, compared to *Shank1*$^{+/+}$ littermate controls (genotype: $F_{1,19}=5.237$, $p = 0.034$; hemisphere:

$F_{1,19}$=67.055, $p < 0.001$; genotype x hemisphere: $F_{1,19}$=2.344, $p = 0.142$; Figure 5c). No such difference in BDNF protein expression was seen under baseline conditions in an independent cohort of mice not exposed to Novel Object Recognition (*Shank1*$^{+/+}$: 0.221 ± 0.030 pg/μg protein; *Shank1*$^{-/-}$: 0.230 ± 0.011 pg/μg protein; genotype: $F_{1,15}$=0.115, $p = 0.739$; hemisphere: $F_{1,15}$=114.977, $p < 0.001$; genotype x hemisphere: $F_{1,15}$=0.519, $p = 0.482$). While in both genotypes BDNF protein expression was higher in the left than in the right hemisphere, Pearson's correlation analysis further revealed that BDNF protein concentrations from the left and right hemispheres were significantly correlated with one another in *Shank1*$^{+/+}$ mice ($r = 0.753$, $p = 0.008$) but not in *Shank1*$^{-/-}$ mice ($r = 0.113$, $p = 0.678$; Figure 5d). Finally, epigenetic regulation of BDNF expression in the hippocampus was found to be affected by genotype. While H4 acetylation did not differ between genotypes ($F_{1,26}$=0.218, $p = 0.645$), H3 acetylation was clearly higher in *Shank1*$^{-/-}$ mice, compared to *Shank1*$^{+/+}$ littermate controls ($F_{1,26}$=5.325, $p = 0.029$; Figure 5e), in line with the observed increase in hippocampal BDNF protein levels detected in *Shank1*$^{-/-}$ mice. Altered epigenetic regulation was specifically observed for *Bdnf* but not the other genes evaluated, i.e. *Il2*, *Il6* and *Tnf-α* (H3 acetylation: $F_{1,26}$=2.937, $p = 0.098$; $F_{1,26}$=0.010, $p = 0.920$; and $F_{1,26}$=0.111, $p = 0.742$; respectively; H4 acetylation: $F_{1,26}$=1.868, $p = 0.183$; $F_{1,26}$=1.782, $p = 0.194$; and $F_{1,26}$=1.470, $p = 0.236$; respectively; Supporting Information Figure 1).

4 | DISCUSSION

ASD is among the most heritable neuropsychiatric conditions, and while available evidence points to a complex set of genetic factors, the *SHANK* gene family has emerged as one of the most promising candidates (Bourgeron, 2015; de la Torre-Ubieta et al., 2016; Guilmatre et al., 2014). Genetic *Shank* mouse models display behavioral phenotypes with relevance to all diagnostic criteria of ASD, that is, persistent deficits in social communication/interaction, together with restricted/repetitive patterns of behavior (American Psychiatric Association, 2013). Recent examples demonstrate that this genetic approach helps to explore underlying neurobiological mechanisms and is thus guiding the identification of novel targets for treatment (Jiang & Ehlers, 2013; Santini & Klann, 2014; Schmeisser & Verpelli, 2016; Yoo et al., 2013). In this study, we focused on deficits in socio-cognitive processes and potential underlying mechanisms in the *Shank1* knockout mouse model for ASD (Hung et al., 2008; Silverman et al., 2011; Sungur et al., 2014, 2016; Wöhr, 2014; Wöhr et al., 2011).

Social approach behavior was evident in all experimental conditions. As juveniles and adults, all genotypes showed intact social motivation as they preferred to sniff the *animal* over the *object*. Our results are at variance with those of Silverman et al. (2011), who employed the same *Shank1* knockout mouse model for ASD in a very similar paradigm and reported impaired social approach behavior, yet lack of sociability was evident in all genotypes, including wildtype littermate controls. It is worth noting that in this study mixed C57BL/6J/129SvJae background mice, the genetic background on which the *Shank1* mutation was bred, displayed intact social approach behavior, indicating that the deficits observed were not dependent on background strain. In the same study, Silverman et al. (2011) did not obtain evidence for genotype effects on direct reciprocal social interaction behavior, consistent with our present findings.

In the Social Recognition paradigm, juvenile mice were able to discriminate the *novel* over the *familiar* stimulus mouse irrespective of genotype, whereas in adults sex-dependent genotype effects were evident. Specifically, in adult males, no evidence for genotype differences in social recognition memory was obtained. However, effects were much weaker than in juveniles and thus no significant preferences for the *novel* stimulus mouse were seen at the level of individual genotypes, possibly due to a general lack of interest or stress caused by the presence of two unrelated males. In females, in contrast, genotype-dependent differences were evident. *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ females showed preference for the *novel* stimulus mouse, whereas in *Shank1*$^{-/-}$ mice the difference in time spent sniffing the *novel* over the *familiar* stimulus mouse did not reach a statistical significance, indicating a deficit in social recognition memory caused by *Shank1* deletion. The latter is consistent with a failure to learn from social experiences in adult male *Shank1*$^{-/-}$ mice reported before. Wöhr et al. (2011) found that adult male *Shank1*$^{-/-}$ mice do not adjust the emission of ultrasonic vocalizations in response to female urine depending on their prior experience with females.

While social recognition was only mildly affected by genotype, our data indicate that juvenile *Shank1*$^{-/-}$ mice are severely impaired in the Novel Object Recognition paradigm, in stark contrast to *Shank1*$^{+/+}$ and *Shank1*$^{+/-}$ littermate controls. This was reflected by a complete lack of preference for the *novel* over the *familiar* object; an effect replicated in independent mouse cohorts. Importantly, these deficits were not due to impairments in discriminatory abilities, since juvenile *Shank1*$^{-/-}$ mice displayed a clear preference for the *unique* object when exposed to three *identical* and one *unique* object at the same time. Consistent with the data obtained in juvenile mice, object recognition memory was dependent on the availability of SHANK1 in adult male mice and severely impaired in adult *Shank1*$^{+/-}$ and *Shank1*$^{-/-}$ males. In adult females, however, evidence for intact object recognition memory was obtained irrespective of genotype, yet the preference for the *novel* object was clearly lower in *Shank1*$^{-/-}$ females. The weaker genotype effect in females is consistent with human data suggesting reduced penetrance in females. In fact, Sato et al. (2012) provided evidence that *SHANK1* deletions cause ASD in males but not females - the first example of autosomal sex-limited expression in ASD. Females who are carriers of a *SHANK1* deletion might be protected by a yet unidentified mechanism, possibly related to sex chromosomes, hormonal changes, or other sex-specific factors (Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013; but see: Wang et al., 2016).

Our findings thus extend the knowledge on cognitive phenotypes displayed by the *Shank1* mouse model for ASD in an age- and sex-dependent manner. Previous studies applying cognitive tasks focused on adult male *Shank1*$^{-/-}$ mice only and reported an aberrant cognitive phenotype characterized by impaired contextual but intact cued fear-

conditioning. Moreover, in the radial-arm-maze task to assess spatial memory, male *Shank1*$^{-/-}$ mice, compared to *Shank1*$^{+/+}$ controls, showed steeper learning curves and reached better performance levels with fewer reference memory errors and virtually absent working memory errors. Enhanced learning capabilities were seen not only during initial training but also reversal learning. However, *Shank1*$^{-/-}$ mice were unable to retain the learning enhancement in the long-term, regressing to reference memory performance levels of *Shank1*$^{+/+}$ controls following a 28 days retention interval (Hung et al., 2008).

While the integration of the present findings into this complex set of results is challenging, our results are consistent with the vast majority of object recognition memory studies in *Shank2* and *Shank3* mouse models for ASD (Berkel et al., 2012; Schmeisser et al., 2012; Wang et al., 2011; Won et al., 2012; Yang et al., 2012). For instance, Schmeisser et al. (2012) assessed object recognition memory in *Shank2* mutants. While all genotypes displayed a preference for the *novel* object, this preference was clearly less pronounced in *Shank2*$^{-/-}$ mice. The reduction in preference is remarkable since a relatively low-demanding version of the test was applied, with a short delay of a couple of seconds between object acquisition and recognition trials. It is thus tempting to speculate that no preference would have been detected in *Shank2*$^{-/-}$ mice with longer delays. By using an approach very similar to ours, Yang et al. (2012) observed a complete lack of object recognition memory in *Shank3*$^{e4-9}$ mutants, with *Shank3*$^{-/-}$ mice exploring *novel* and *familiar* objects about the same amount of time after a 30 min delay.

The hippocampus is a key brain structure involved in learning and memory, including object recognition memory (Antunes & Biala, 2012; Dere, Huston, & De Souza Silva, 2007) and social recognition memory (Hitti & Siegelbaum, 2014; Piskorowski et al., 2016). It is strongly implicated in ASD (Li et al., 2016; Radwan, Dvorak, & Fenton, 2016). Interestingly, *Shank1* mRNA levels are particularly high in the hippocampus (Peça et al., 2011). Specifically, by means of *in situ* hybridization, strong *Shank1* mRNA expression was detected in the molecular layer (Boeckers et al. 2004; Zitzer, Honck, Bachner, Richter, & Kreienkamp, 1999) and all three *Shank* mRNAs were found to be expressed in the neuropil layer of the CA1 region, yet *Shank1* mRNA had the highest dendritic expression (Epstein et al. 2014). This enrichment in the neuropil, compared to soma, is viewed as evidence for local translation being an essential source of SHANK1 protein at the synapse during activity-dependent plasticity (Sala et al., 2015). Interestingly, Studtmann et al. (2014) identified a non-canonical initiation site necessary for efficient translation of dendritically localized *Shank1* mRNA. In *Shank1*$^{-/-}$ mice, Hung et al. (2008) described altered PSD protein composition, with reduced levels of SHANK, Homer, and GKAP/SAPAP, together with smaller dendritic spines and synapses in the hippocampus, resulting in a weakening of excitatory synaptic transmission. Filice, Vörckel, Sungur, Wöhr, and Schwaller (2016) further found the calcium buffer parvalbumin being strongly decreased in *Shank1*$^{-/-}$ mice, particularly in brain regions with high SHANK1 expression under wildtype conditions. Parvalbumin is involved in maintaining the excitation/inhibition balance in the brain, with reductions being associated, at the neuronal level, with enhanced inhibition, and, at the behavioral level, with cognitive deficits, including reversal learning impairments (Wöhr et al., 2015).

Here, we show that, after Novel Object Recognition, hippocampal BDNF protein expression is higher in *Shank1*$^{-/-}$ mice, compared to *Shank1*$^{+/+}$ littermates. No such difference in BDNF levels was seen in mice not exposed to Novel Object Recognition. Interestingly, BDNF concentrations from left and right hemispheres were significantly correlated with one another in *Shank1*$^{+/+}$ but not *Shank1*$^{-/-}$ mice; possibly related to alterations in brain development typically seen in ASD mouse models (Ellegood et al., 2015) and human ASD (Amaral, Schumann, & Nordahl, 2008). BDNF regulates neuronal structure and function (Barde, Edgar, & Thoenen, 1982; Leibrock et al., 1989). It modulates hippocampal long-term potentiation (Korte et al., 1995) and thus learning and memory, including fear-conditioning (Endres & Lessmann, 2012). Consistent with the present findings, BDNF expression in the hippocampus was found to be negatively correlated with object recognition memory (Muñoz et al., 2010) and BDNF blockade had no detrimental effect on task performance when short delays of several minutes between object acquisition trial and object recognition trial were applied (Callaghan & Kelly, 2013).

In line with the idea that dysregulation of activity-dependent signaling pathways in neurons plays a prominent role in ASD etiology (Ebert & Greenberg, 2013), BDNF was suggested to be involved (Nickl-Jockschat & Michel, 2011), and its overexpression has emerged as potential factor in ASD pathophysiology (Abdallah et al., 2013; Connolly et al., 2006; Correia et al., 2010; Miyazaki et al., 2004; Nelson et al., 2001). However, despite a possible link between SHANK and BDNF (Bourgeron, 2015; Koh, Lim, Byun, & Yoo, 2014), BDNF levels have not been investigated in any of the available *Shank* models so far, in contrast to other ASD mouse models. For example, in the valproic-acid (VPA) model, a transient increase in *Bdnf* mRNA and protein levels was observed in the fetal brain after exposure to VPA, a widely used anti-epileptic drug also acting as histone deacetylase (HDAC) inhibitor (Almeida, Roby, & Krueger, 2014). Similarly, in the *Fmr1* knockout model for fragile X syndrome, BDNF protein levels were decreased in the cortex but increased in the hippocampus (Louhivuori et al., 2011). Besides, BDNF-overexpressing mice were reported to display memory deficits (Papaleo et al., 2011), consistent with the object recognition impairments seen in BDNF-overexpressing *Shank1*$^{-/-}$ mice.

It is of great interest to unveil how BDNF expression is upregulated in *Shank1*$^{-/-}$ mice. In addition to protein levels, we therefore investigated changes in the epigenetic regulation of hippocampal BDNF expression. The structure of the *Bdnf* gene is complex as it consists of eight 5′-noncoding promoter exons which are spliced to one common 3' exon coding for the BDNF preprotein amino acid sequence and results in different *Bdnf* transcripts. These different *Bdnf* promoters are used to achieve precise temporal and spatial control of BDNF production (Timmusk et al., 1993). Transcription from these promoters is tightly regulated by DNA methylation as well as histone modifications. While CpG methylation of *Bdnf* promoters is linked to silencing of the gene, acetylation of the histones H3 and H4 mediates transcriptional activation (Boulle et al., 2012; Tessarz & Kouzarides, 2014). Consistent

with the observed increase in hippocampal BDNF protein levels, we detected an enrichment of H3 but not H4 acetylation at the *Bdnf* promoter1 in *Shank1*$^{-/-}$ mice, compared to *Shank1*$^{+/+}$ littermates. Of note, this alteration in epigenetic regulation was specifically observed for *Bdnf* but not for *Il2*, *Il6*, or *Tnf-α*, which are also implicated in cognitive functioning (Balschun et al., 2004; del Rey, Balschun, Wetzel, Randolf, & Besedovsky, 2013; Petitto, McNamara, Gendreau, Huang, & Jackson, 1999) and, particularly *Il6*, linked to ASD (Masi et al., 2015), indicating that differences in the epigenetic regulation of BDNF between *Shank1*$^{-/-}$ mice and *Shank1*$^{+/+}$ littermates are not due to a general effect. These findings go in line with previous reports showing varying histone signatures at *Bdnf* promoters, yet linking H3 acetylation of promoter1 with increased *Bdnf* mRNA expression (Boulle et al., 2012; Bredy et al., 2007). Active demethylation within the regulatory region of the *Bdnf* gene after depolarization of neurons has been described to induce BDNF production (Martinowich et al., 2003). Whether this mechanism acts in support of the detected permissive histone marks needs to be further evaluated. While our data adds to growing evidence linking *SHANK* and ASD through epigenetic modifications (Uchino & Waga, 2013), suggesting epigenetic regulation as a potential therapeutic target, e.g. with HDACs being a primary target or by administration of the methyl donor S-adenosylmethionine (Szyf, 2015), alternate pathways might be involved, including compensatory mechanisms via the synaptic zinc-metalloproteinase-BDNF axis (Koh et al., 2014; but see: Grabrucker, 2014).

In humans, cognitive impairment is common in ASD, and ~70% of individuals with ASD suffer from mental retardation (Fombonne, 1999). Interestingly, mutations of *SHANK* genes were detected in the whole spectrum, with a gradient of severity in intellectual disability (Leblond et al., 2014). Specifically, *SHANK3* mutations were mainly found in individuals with ASD combined with moderate to severe mental retardation, *SHANK2* mutations in cases with ASD and mild intellectual disability, and *SHANK1* mutations mostly in individuals with ASD and normal IQ. Despite normal IQ-values in most individuals with ASD carrying a *SHANK1* deletion, cognitive processes might still be affected due to immature neuronal networks with reduced numbers of large spine heads (Sala et al., 2015), consistent with *in-vitro* studies demonstrating that overexpression of *Shank1* leads to increased spine size (Sala et al., 2001). In fact, evidence for a role of *SHANK1* in learning and memory was first provided by a study on *SHANK1* promoter variants showing that the rs3810280 T-allele variant is associated with reduced auditory working memory capacity (Lennertz et al., 2012). Moreover, very recently Wang et al. (2016) reported a *SHANK1* mutation in an individual with ASD and intellectual disability, strongly supporting a link between *SHANK1* and cognitive functioning, and in line with our mouse findings.

5 | CONCLUSIONS

Together, our findings indicate that *Shank1* deletions lead to an aberrant cognitive phenotype characterized by severe impairments in object recognition memory and increased hippocampal BDNF levels, possibly due to epigenetic modifications. This result supports the link between ASD and intellectual disability, and suggests epigenetic regulation as a potential therapeutic target.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to M.W. (DFG; WO 1732/1–1) and a young investigator grant from the Faculty of Psychology, Philipps-University of Marburg, to M.W. (PUM; WO 2012/1). The authors wish to thank Jacqueline Crawley, University of California Davis School of Medicine, and the Howard Hughes Medical Institute investigators Albert Hung and Morgan Sheng for providing the *Shank1* mouse line. The authors also wish to thank Elena Andres and Tobias Redecker for their help in this project.

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SUPPORTING INFORMATION

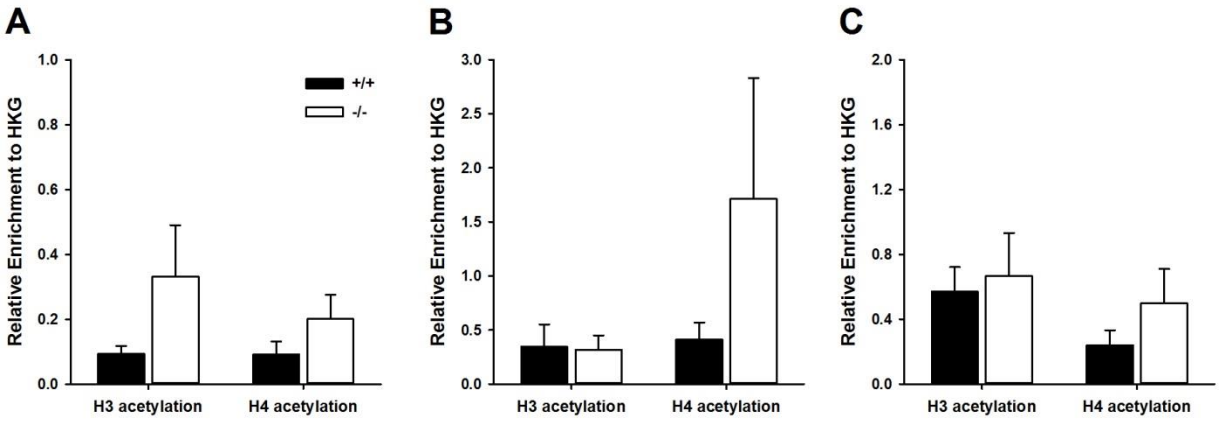
Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Sungur A. Özge, Jochner MCE, Harb H, et al. Aberrant cognitive phenotypes and altered hippocampal BDNF expression related to epigenetic modifications in mice lacking the post-synaptic scaffolding protein SHANK1: Implications for autism spectrum disorder. *Hippocampus*. 2017;00:1–14. https://doi.org/10.1002/hipo.22741

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES

Supplementary Figure 1:



SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Interleukin 2 (*Il2*), interleukin 6 (*Il6*) and tumor necrosis factor-alpha (*Tnf-α*) histone acetylation levels in juvenile *Shank1* mice. Histone 3 (H3) and 4 (H4) acetylation levels are depicted by enrichment levels (relative to housekeeping gene; HKG). There were no differences in acetylation levels for (A) *Il2*, (B) *Il6*, and (C) *Tnf-α* in the hippocampi of the *Shank1*$^{-/-}$ mice (white bar) in comparison to *Shank1*$^{+/+}$ mice (black bar). Data are presented as means+SEM.

Study II: Early communication deficits in the Shank1 knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context

RESEARCH ARTICLE

Early Communication Deficits in the *Shank1* Knockout Mouse Model for Autism Spectrum Disorder: Developmental Aspects and Effects of Social Context

A. Özge Sungur, Rainer K.W. Schwarting, and Markus Wöhr

Alterations in *SHANK* genes were repeatedly reported in autism spectrum disorder (ASD). ASD is a group of neurodevelopmental disorders diagnosed by persistent deficits in social communication/interaction across multiple contexts, with restricted/repetitive patterns of behavior. To date, diagnostic criteria for ASD are purely behaviorally defined and reliable biomarkers have still not been identified. The validity of mouse models for ASD therefore strongly relies on their behavioral phenotype. Here, we studied communication by means of isolation-induced pup ultrasonic vocalizations (USV) in the *Shank1* mouse model for ASD by comparing *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate controls. The first aim of the present study was to evaluate the effects of *Shank1* deletions on developmental aspects of communication in order to see whether ASD-related communication deficits are due to general impairment or delay in development. Second, we focused on social context effects on USV production. We show that *Shank1*$^{-/-}$ pups vocalized less and displayed a delay in the typical inverted U-shaped developmental USV emission pattern with USV rates peaking on postnatal day (PND) 9, resulting in a prominent genotype difference on PND6. Moreover, testing under social conditions revealed even more prominently genotype-dependent deficits regardless of the familiarity of the social context. As communication by definition serves a social function, introducing a social component to the typically nonsocial test environment could therefore help to reveal communication deficits in mouse models for ASD. Together, these results indicate that *SHANK1* is involved in acoustic communication across species, with genetic alterations in *SHANK1* resulting in social communication/interaction deficits. ***Autism Res*** *2016, 9: 696–709*. © 2015 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: animal model; postsynaptic density; neurodevelopmental disorders; autism; communication; ultrasonic vocalization; social context

Introduction

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorders with a strong genetic component. ASD is diagnosed by persistent deficits in social communication/interaction across multiple contexts, with restricted/repetitive patterns of behavior [American Psychiatric Association, 2013]. While the exact causes of ASD remain unknown, there is evidence for alterations in various molecular pathways involved in glutamatergic neurotransmission, particularly synapse formation and functioning, likely resulting in an imbalance between inhibitory and excitatory synaptic currents [Bourgeron, 2009; Peça & Feng, 2012; Tang et al., 2014]. Expression of *SHANK* genes is a key component of these ASD-associated pathways as the protein products are enriched in the postsynaptic density (PSD) of glutamatergic neurons. The unique and highly conserved domains grant specificity for protein–protein interactions, suggesting an important organizational role at the PSD [Ting, Peça, & Feng, 2012], with the three SHANK family members, SHANK1, SHANK2, and SHANK3, serving as master scaffolding proteins by linking to the actin cytoskeleton and interacting with AMPAR, NMDAR, mGluR, and neuroligins through GKAP/SAPAP and Homer [Kim & Sheng, 2004; Naisbitt et al., 1999].

Alterations in *SHANK* genes, such as deletions, duplications, and coding mutations, were repeatedly reported in ASD [Guilmatre, Huguet, Delorme, & Bourgeron, 2014; Leblond et al., 2014]. Specifically, Durand et al. [2007] first described mutations in *SHANK3*, with subsequent studies reporting mutations in *SHANK1* [Sato et al., 2012], *SHANK2* [Berkel et al., 2010; Leblond et al., 2012; Pinto et al., 2010], and *SHANK3* [Gauthier et al., 2009; Moessner et al., 2007] in cases of ASD and

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Conflict of Interest: No conflict of interest.
Received March 10, 2015; accepted for publication August 21, 2015

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Published online 30 September 2015 in Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/aur.1564

schizophrenia patients with ASD traits. Moreover, *SHANK3* haploinsufficiency has been found in patients affected by the Phelan-McDermid 22q13 deletion syndrome, a form of mental retardation often paralleled by ASD features, such as impaired language acquisition [Bonaglia et al., 2001; Manning et al., 2004; Phelan et al., 2001; Wilson et al., 2003]. Up to now, more than 900 patients with genetic alterations in *SHANK* genes were identified, with the *SHANK* gene family being the primary gene family implicated in ASD [Leblond et al., 2014]. Furthermore, valproate treatment, probably the best established environmental risk factor for ASD [Moore et al., 2000] and being associated with speech delay [Dean et al., 2002], was shown to reduce *Shank* mRNA levels in cortical brain areas in mice [De Bartolomeis, Tomasetti, Cicale, Yuan, & Manji, 2012]. Consequently, various genetic *Shank* models were generated, including *Shank1* [Hung et al., 2008], *Shank2* [Schmeisser et al., 2012; Won et al., 2012], and *Shank3* null mutant mice [Kouser et al., 2013, Peça et al., 2011; Wang et al., 2011; Yang et al., 2012] that allow to study neurobiological factors underlying ASD and to test novel pharmacological treatments for their efficacy in reversing ASD-related behavioral phenotypes.

To date, the diagnostic criteria for ASD are purely behaviorally defined and reliable biomarkers have still not been identified. The validity of genetic mouse models for ASD therefore strongly relies on their behavioral phenotype. For this reason, deep and longitudinal behavioral phenotyping constitutes the principle component for current translational research, which strongly depends on sensitive behavioral test paradigms with relevance to each symptom category [Silverman, Yang, Lord, & Crawley, 2010]. Within the last decade, a comprehensive set of behavioral assays for detecting deficits in mouse social and communication behavior across multiple contexts was developed [Bishop & Lahvis, 2011; Silverman et al., 2010; Wöhr & Scattoni, 2013]. Typically, ultrasonic vocalizations (USV) are used for assessing communication deficits in mouse models for ASD [Scattoni, Crawley, & Ricceri, 2009; Wöhr, 2014]. Mice emit distinct types of USV depending on developmental stage and social context: isolation-induced USV in pups, and interaction-induced USV in juveniles and adults. USV emitted by pups are mainly driven by separation from mother and littermates [Zippelius & Schleidt, 1956]. Such isolation-induced USV often increase during the first week of life and decrease thereafter, giving rise to an inverted U-shaped pattern of call emission. In mouse models for ASD, this inverted U-shaped developmental call emission pattern is commonly delayed and/or distorted [Chadman et al., 2008; Ey et al., 2013; Mosienko, Beis, Alenina, & Wöhr, 2015; Schmeisser et al., 2012]. Besides developmental factors, social odors play an important role in modulating pup USV emission [Branchi, Santucci, Vitale, & Alleva, 1998]. This is particularly relevant for mouse models for ASD, as it is believed that ASD is characterized by deficits in the processing of social context information [American Psychiatric Association, 2013]. For instance, it has been repeatedly shown that mouse pups emit fewer USV when exposed to nest odor than to clean bedding [D'Amato & Cabib, 1987; Moles, Kieffer, & D'Amato, 2004; Wöhr, 2015]. Emphasizing the relevance of such a calming response for ASD mouse models, no such effect was seen in the μ-opioid knockout mouse model for ASD [Moles et al., 2004], which displays a variety of ASD-related behavioral deficits, including communication deficits [Oddi, Crusio, D'Amato, & Pietropaolo, 2013]. Furthermore, also the well-established BTBR T+tf/J mouse model for ASD [Blanchard et al., 2012; Meyza et al., 2013] showed a limited ability and/or reduced motivation to adjust to different social contexts [Wöhr, 2015].

The first *Shank* mouse model for ASD was generated by Hung et al. [2008] via disruption of the *Shank1* gene through deletion of exons 14 and 15, leading to a complete knockout of all SHANK1 proteins. Behavioral studies revealed that *Shank1* null mutants manifest increased anxiety-related behavior, impaired contextual fear memory, and enhanced acquisition but impaired retention of spatial learning, possibly resembling the aberrant cognitive phenotype present in some ASD cases [Hung et al., 2008; Silverman et al., 2011]. While social interaction behavior was reported to be unchanged [Silverman et al., 2011], evidence for lifelong communication deficits was provided [Wöhr, Roullet, Hung, Sheng, & Crawley, 2011]. Specifically, Wöhr et al. [2011] found reduced isolation-induced USV in pups, together with early developmental deficits. They further observed a lack of social modulation of adult male USV in response to female urine and reduced scent marking behavior in *Shank1* null mutants, indicating a failure to learn from social experiences. *Shank1* null mutants also displayed strong alterations in repetitive behavior, with elevated self-grooming behavior, particularly when tested in a social context [Sungur, Vörckel, Schwarting, & Wöhr, 2014].

Currently, little is known about the mechanisms underlying the ultrasonic communication deficits displayed by *Shank1* null mutant pups and whether such deficits persist across multiple social contexts has not been tested yet. The first aim of the present study was therefore to evaluate the effects of *Shank1* deletions on developmental aspects of communication to see whether ASD-related communication deficits are due to general impairment or delay in development. Second, we focused on the effects of social context on USV production, by comparing clean bedding, home cage bedding, and male cage bedding conditions.

Materials and Methods

Animals and Housing

Isolation-induced USV were assessed in *Shank1*$^{-/-}$ null mutant mouse pups with a targeted replacement of exons 14 and 15 encoding almost the entire PDZ domain and were compared to *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{+/+}$ wildtype littermate control mice. Mice were obtained from mutant lines originally generated by Hung et al. [2008] on two independent background strains: C57BL/6J and 129SvJae. The two lines were crossed for at least three generations to produce a mixed C57BL/6J/129SvJae background for the *Shank1* mutation, consistent with other studies focusing on this *Shank1* mutant [Hung et al., 2008; Silverman et al., 2011; Sungur et al., 2014; Wöhr et al., 2011]. Using a heterozygous breeding protocol, *Shank1*$^{+/-}$ males and females were bred in a conventional vivarium at the Biomedical Research Center of the Philipps-University of Marburg, Germany. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. Bedding and a wooden board were provided in each cage. Standard rodent chow and water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle (lights on: 06:00 hr) at ~22°C (40–50% humidity). All procedures were approved by the ethical committee of the local government (Regierungspräsidium, Gießen, Germany).

General Overview

Two experiments on isolation-induced USV were conducted, focusing on developmental aspects (Experiment I) and the effects of social context (Experiment II). In both experiments, *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice were compared. Testing was conducted during the light phase of the 12:12 hr light/dark cycle. Pups were tested only once to avoid carry-over effects. They were identified by paw tattoo and tail samples for genotyping were taken after completion of behavioral experiments [for details: Sungur et al., 2014]. Experimenters were blind to genotypes during data acquisition and analysis.

Independent Variables

Experiment I: developmental aspects. In Experiment I, a design with three independent factors was used, namely genotype, sex, and development, in order to study developmental aspects of isolation-induced USV in a genotype- and sex-dependent manner. To this aim, mouse pups from different litters were tested on PND3, PND6, PND9, or PND12, using clean bedding.

Experiment II: effects of social context. In Experiment II, a design with three independent factors was used, namely genotype, sex, and social context, in order to study the effects of social context on isolation-induced USV in a genotype- and sex-dependent manner. To this aim, mouse pups from different litters were tested on PND9, using clean bedding, home cage bedding, or male cage bedding obtained from a cage with unfamiliar adult C57BL6/J males. Home cages and male cages used to obtain soiled bedding material were not cleaned for two days prior testing in order to expose mouse pups to sufficiently distinct odor stimuli. Soiled bedding contained feces. Pups were tested on PND9 since in Experiment I no genotype differences on that PND were detected under clean bedding conditions.

Dependent Variables

Isolation-induced USV—Recording. For inducing isolation-induced USV, pups were isolated from their mother and littermates for 10 min under room temperature (20–23°C). Pups were removed individually from the nest at random and gently placed into a glass isolation container (10 × 8 × 6 cm; open surface), containing clean or soiled bedding material depending on the experiment (Experiment I: clean bedding material was used throughout; Experiment II: clean bedding, home cage bedding, and male cage bedding were compared). The isolation container was surrounded by a sound attenuating box (21 × 21 × 21 cm) made of Styrofoam (thickness of walls: 6 cm). USV emission was monitored by an UltraSoundGate Condenser Microphone CM16 sensitive to frequencies of 15–180 kHz (flat frequency response between 25 and 140 kHz; ±6 dB; Avisoft Bioacoustics, Berlin, Germany) placed in the roof of the sound attenuating box, 22 cm above the floor. It was connected via an UltraSoundGate 416 USGH audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz (16 bit) by Avisoft RECORDER (version 2.97). Prior to each test, behavioral equipment was cleaned using 0.1% acetic acid solution.

Isolation-induced USV—Analysis. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 5.20) and a fast Fourier transform was conducted (512 FFT length, 100% frame, Hamming window, and 75% time window overlap), resulting in spectrograms with 488 Hz of frequency and 0.512 ms of time resolution. Call detection was provided by an automatic threshold-based algorithm (amplitude threshold: −40 dB; hold time: 10 ms; high-pass filter: 30 kHz). Accuracy of call detection was verified by an experienced user. When necessary, missed calls were marked

manually to be included in the automatic parameter analysis. In Experiments I and II, total numbers of USV were calculated for the entire session and, in Experiment II, in 60 sec time bins to visualize the time course of the USV response. In Experiment I, additional parameters included latency to start calling, call duration, peak frequency, peak amplitude, and frequency modulation. Finally, the temporal organization of isolation-induced USV emission was assessed through sequential analyses and call subtypes were determined by means of density plots (for details: Supporting Information).

Body temperature and weight. After the 10 min isolation period, body temperature and weight were determined in pups from Experiment I. For body temperature determination, a Testo 110 thermometer with surface sensor (Testo AG, Lenzkirch, Germany) was used. Body weight was measured using a palmscale (PS6-250; MyWeigh Europe, Hückelhoven, Germany). Finally, somatosensory reflexes were determined (for details: Supporting Information).

Statistical Analysis

For analysis of isolation-induced USV, body temperature, and weight measured in Experiment I, ANOVAs with the between-subjects factors genotype, sex, and PND were calculated to assess genotype differences between *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice. In Experiment II, ANOVAs with the between-subject factors genotype and sex were applied for each of the three different social contexts. In addition, ANOVAs for repeated measures with the between-subject factors genotype and sex and the within-subject factor minute were used to analyze the time course of isolation-induced USV emission. Approximately half of the *Shank1*$^{+/-}$ mice were randomly excluded from both experiments to obtain similar numbers of mice per genotype. ANOVAs were followed by LSD post hoc analysis when appropriate. A *P*-value of <0.050 was considered statistically significant.

RESULTS

Experiment I: Developmental Aspects

Isolation-induced USV—Development. Emission rates of isolation-induced USV changed with development ($F_{3,131} = 20.614$, $P < 0.001$). Specifically, an inverted U-shaped pattern was detected, with call emission rates peaking on PND6. High rates were also detected on PND9, while lower call numbers were found on PND3 and PND12 (Fig. 1A′). An inverse pattern was found for latency to start calling ($F_{3,131} = 11.182$, $P < 0.001$), with latencies being lowest on PND6, i.e. the day with the highest call rate. Call duration gradually decreased with development ($F_{3,131} = 9.970$, $P < 0.001$; Fig. 2A′). In addition, call peak amplitude ($F_{3,131} = 5.212$, $P = 0.002$; Fig. 2B′), peak frequency ($F_{3,131} = 9.408$, $P < 0.001$; Fig. 2C′), and frequency modulation ($F_{3,131} = 6.473$, $P < 0.001$) changed with age. While peak frequency was characterized by a U-shaped developmental pattern, an inverted U-shaped pattern was evident in case of peak amplitude and frequency modulation.

Isolation-induced USV—Genotype. Isolation-induced USV emission rates differed between genotypes ($F_{2,131} = 3.295$, $P = 0.040$; genotype × development: $F_{6,131} = 1.555$, $P = 0.165$). Overall, *Shank1*$^{-/-}$ null mutant mouse pups emitted fewer isolation-induced USV than *Shank1*$^{+/-}$ heterozygous ($P = 0.003$) and *Shank1*$^{+/+}$ wildtype littermate controls ($P = 0.006$), with the latter not differing ($P = 0.799$; Fig. 1A). When comparing genotypes on individual PNDs, no genotype differences were obtained on PND3, PND9, and PND12 (all *P*-values >0.050). However, a strong genotype effect was found on PND6 (genotype: $F_{2,36} = 4.625$, $P = 0.016$; Fig. 1A′). While in *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ controls call rate was highest on PND6 (Fig. 1A′, solid curve), call rate was low in *Shank1*$^{-/-}$ pups on PND6, as compared to *Shank1*$^{+/-}$ ($P = 0.007$) and *Shank1*$^{+/+}$ controls ($P = 0.028$), which did not differ ($P = 0.562$; Fig. 1A′). In *Shank1*$^{-/-}$ pups, call emission peaked on PND9 not PND6, possibly reflecting a delay in early development (Fig. 1A′, dotted curve). Besides call number, peak amplitude was affected by genotype ($F_{2,131} = 4.661$, $P = 0.011$; genotype × development: $F_{6,131} = 1.288$, $P = 0.267$). Overall, *Shank1*$^{-/-}$ pups emitted isolation-induced USV with lower peak amplitudes than *Shank1*$^{+/-}$ ($P = 0.018$) and *Shank1*$^{+/+}$ controls ($P = 0.002$), with the latter not differing ($P = 0.455$; Fig. 2B). When comparing genotypes on individual PNDs, no genotype differences were observed on PND3 and PND6 (all *P*-values >0.050), while prominent genotype effects were detected on PND9 and PND12 ($F_{2,32} = 5.188$, $P = 0.011$ and $F_{2,30} = 4.965$, $P = 0.014$; respectively). On PND9, isolation-induced USV emitted by *Shank1*$^{-/-}$ pups were lower in amplitude than the ones emitted by *Shank1*$^{+/-}$ ($P = 0.017$) and *Shank1*$^{+/+}$ controls ($P = 0.002$), with the latter not differing ($P = 0.375$). On PND12, isolation-induced USV emitted by *Shank1*$^{-/-}$ pups were again lower in amplitude than the ones emitted by *Shank1*$^{+/+}$ controls ($P = 0.017$), yet the difference between *Shank1*$^{-/-}$ and *Shank1*$^{+/-}$ pups was not statistically significant anymore ($P = 0.310$). Also, *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ pups did not differ ($P = 0.122$; Fig. 2B′). No evidence for genotype differences was obtained for the other call parameters, including call duration (Fig. 2A

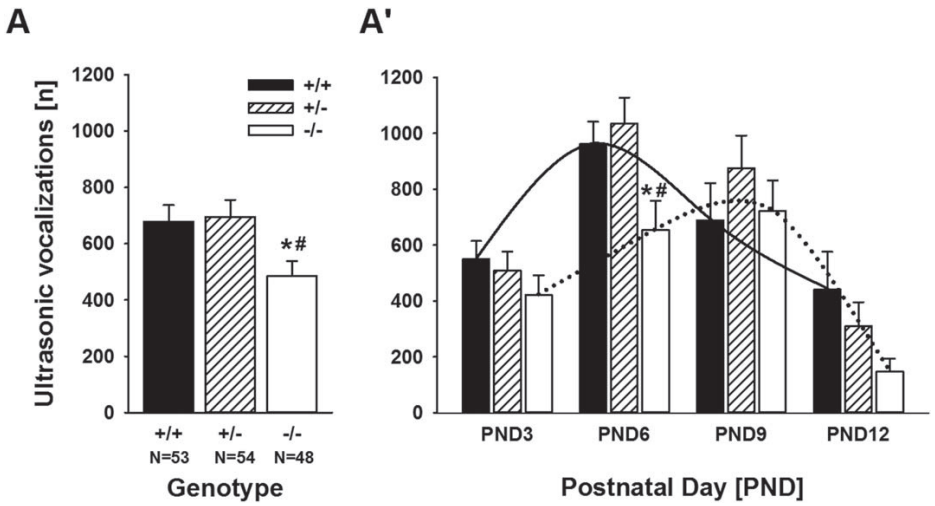

Figure 1. Experiment I: Number of ultrasonic vocalizations (USV) [n] in isolated *Shank1* pups. (A) Total number of USV [n] emitted on average over all four postnatal days [PND]. (A′) Developmental course for the total number of USV emitted on PND3, PND6, PND9, and PND12. Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Solid curve: Developmental pattern of USV emitted by *Shank1*$^{+/+}$ mice; dotted curve: developmental pattern of USV emitted by *Shank1*$^{-/-}$ mice. For the sake of clarity, no curve depicting the developmental pattern of USV emitted by *Shank1*$^{+/-}$ mice was included in the developmental course graph. Group sizes (for individual PNDs): *Shank1*$^{+/+}$ mice: $N_{PND3} = 12$, $N_{PND6}=15$, $N_{PND9}=14$, $N_{PND12} = 12$; *Shank1*$^{+/-}$ mice: $N_{PND3} = 13$, $N_{PND6} = 15$, $N_{PND9} = 13$, $N_{PND12} = 13$; *Shank1*$^{-/-}$ mice: $N_{PND3} = 14$, $N_{PND6} = 12$, $N_{PND9} = 11$, $N_{PND12} = 11$. Data are presented as means + standard errors of the mean. $^{*}P < 0.050$ vs. *Shank1*$^{+/+}$; $^{\#}P < 0.050$ vs. *Shank1*$^{+/-}$.

and A′) and peak frequency (Fig. 2C and C′; all *P*-values >0.050; genotype x development: all *P*-values >0.050).

Isolation-induced USV—Sex. USV numbers differed between males and females in an age-dependent manner ($F_{1,131} = 3.075$, $P = 0.082$; sex × development: $F_{3,131} = 2.907$, $P = 0.037$). While no sex differences were obtained on PND3, PND6, and PND12 (all *P*-values >0.050), a sex effect was found on PND9 ($F_{1,32}=5.695$, $P = 0.023$), with females emitting more isolation-induced USV than males. Besides call number, call duration was affected by sex in an age-dependent manner ($F_{1,131} = 0.126$, $P = 0.723$; sex × development: $F_{3,131} = 2.837$, $P = 0.041$). No sex differences were obtained on PND3, PND9, and PND12 (all *P*-values >0.050), but on PND6 ($F_{1,36} = 7.692$, $P = 0.009$), with females emitting longer isolation-induced USV than males. For the other call parameters, no evidence for sex effects or interactions between sex and genotype was obtained (all *P*-values >0.050; not shown).

Isolation-induced USV—Detailed subtype and temporal analyses. In a subsequent detailed subtype analysis based on 1,000-8,000 calls emitted by *Shank1*$^{-/-}$ pups per PND and 5,000–15,000 calls per PND from *Shank1*$^{+/+}$ controls, clusters of isolation-induced USV were revealed by density plots (Fig. 3). On PND3, one cluster was identified with most USV being characterized by peak frequencies between 50 and 80 kHz in both genotypes. From PND6 on, two clusters were evident. Similar to PND3, most USV were still characterized by peak frequencies between 50 and 80 kHz, yet average peak frequency in this cluster successively decreased with development. This decrease was paralleled by the segregation and formation of a new second cluster of USV with peak frequencies between 80 and 100 kHz, which gradually became more prominent with development. Interestingly, it was consistently more pronounced in *Shank1*$^{-/-}$ than in *Shank1*$^{+/+}$ pups, and on PND12 this cluster was even more prominent than the first cluster between 50 and 80 kHz, which was not the case in *Shank1*$^{+/+}$ controls. An additional detailed temporal analysis by means of sequential correlational analyses of the durations of subsequent isolation-induced USV further indicated that the call emission pattern is not random in *Shank1*$^{+/+}$ controls, since the durations of given USV could be predicted by the durations of the previous ones (N-1) and to a lesser extent also by the ones two (N-2) and three (N-3) before (Fig. 4). Remarkably, such a nonrandom pattern was already seen on PND3, with temporal organization gradually decreasing with development. A very similar development of temporal organization was seen in *Shank1*$^{-/-}$ pups.

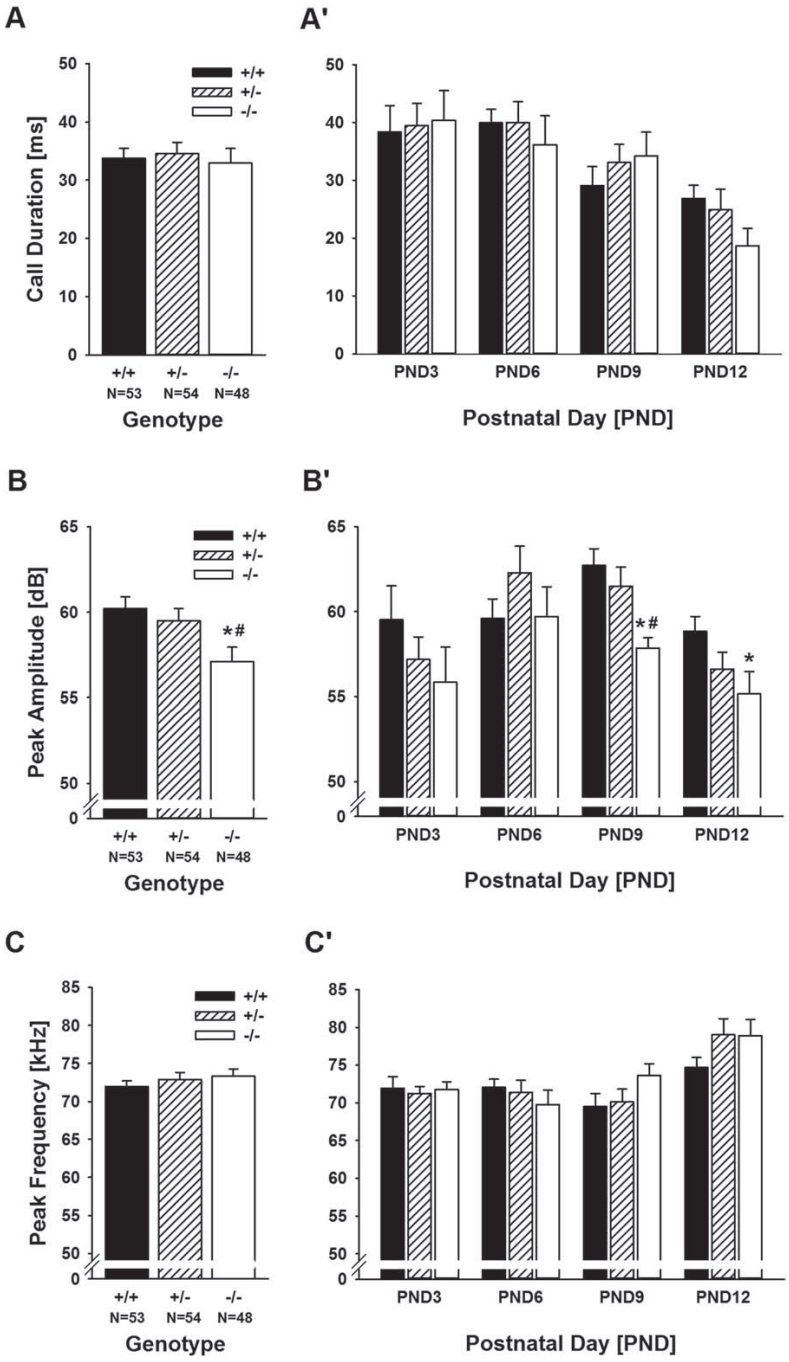

Figure 2. Experiment I: Acoustic characteristics of ultrasonic vocalizations (USV) in isolated *Shank1* pups. (A) Duration (in milliseconds [ms]), (B) peak amplitude (in decibel [dB]), and (C) peak frequency (in kilohertz [kHz]) of USV emitted on average over all four postnatal days [PND]. Developmental course for the (A′) duration, (B′) peak amplitude, and (C′) peak frequency of calls emitted on PND3, PND6, PND9, and PND12. Black bar: $Shank1^{+/+}$ wildtype littermate control mice; striped bar: $Shank1^{+/-}$ heterozygous mice; white bar: $Shank1^{-/-}$ null mutant mice. Group sizes (for individual PNDs): $Shank1^{+/+}$ mice: $N_{PND3} = 12$, $N_{PND6} = 15$, $N_{PND9} = 14$, $N_{PND12} = 12$; $Shank1^{+/-}$ mice: $N_{PND3} = 13$, $N_{PND6} = 15$, $N_{PND9} = 13$, $N_{PND12} = 13$; $Shank1^{-/-}$ mice: $N_{PND3} = 14$, $N_{PND6} = 12$, $N_{PND9} = 11$, $N_{PND12} = 11$. Data are presented as means + standard errors of the mean. $^{*}P < 0.050$ vs. $Shank1^{+/+}$; $^{\#}P < 0.050$ vs. $Shank1^{+/-}$.

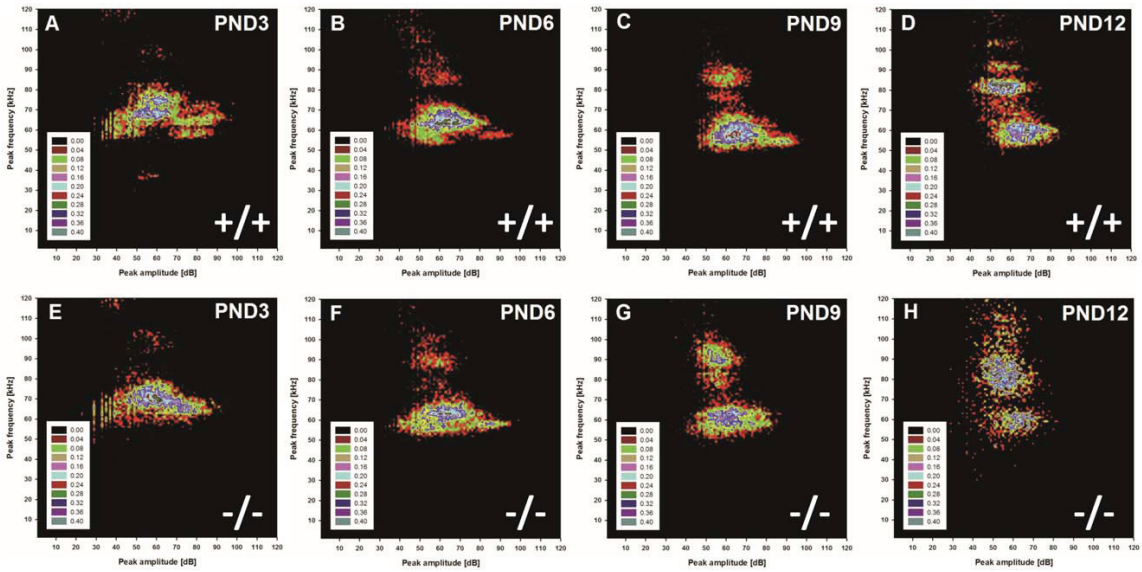

Figure 3. Experiment I: Distribution of individual ultrasonic vocalizations (USV) in isolated *Shank1* pups. Density plots depicting the distribution of individual calls depending on peak frequency (in kilohertz [kHz]) and peak amplitude (in decibel [dB]) in *Shank1*$^{+/+}$ wildtype littermate control mice on individual postnatal days [PND], namely (A) PND3, (B) PND6, (C) PND9, and (D) PND12, and in *Shank1*$^{-/-}$ null mutant mice on (E) PND3, (F) PND6, (G) PND9, and (H) PND12. Color coding reflects frequencies as percentages.

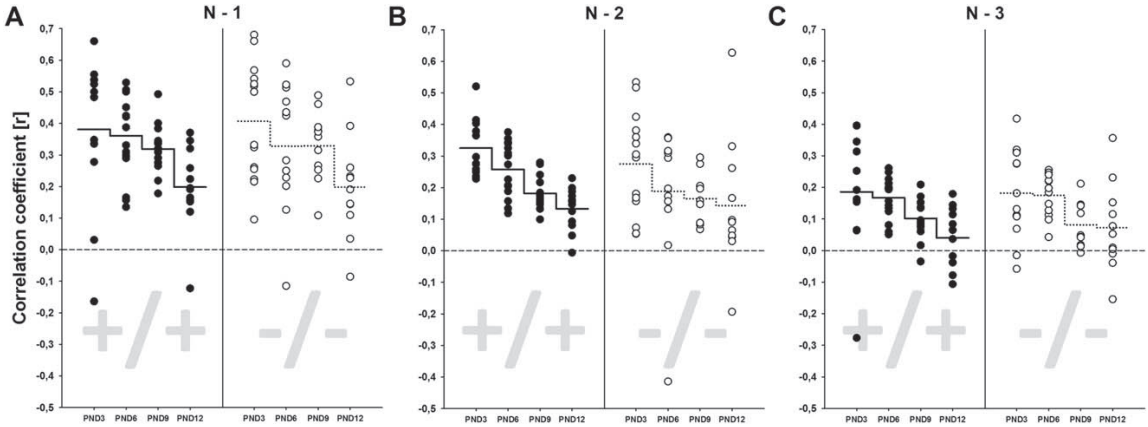

Figure 4. Experiment I: Temporal analysis of the correlations between durations of subsequent ultrasonic vocalizations (USV) in isolated *Shank1* pups. Point plots depicting the sequential organization of USV by means of correlations (correlation coefficient [r]) between durations of isolation-induced USV with those of (A) the previous ones (N-1), (B) the ones two before (N-2) and (C) the ones three before (N-3) on individual postnatal days [PND], namely PND3, PND6, PND9, and PND12. Black circles: *Shank1*$^{+/+}$ wild-type littermate control mice; white circle: *Shank1*$^{-/-}$ null mutant mice. Step-wise patterns of the sequential organization of mean correlation coefficients are depicted by step plots. Solid line: mean correlation coefficients in *Shank1*$^{+/+}$ mice; dotted line: mean correlation coefficients in *Shank1*$^{-/-}$ mice.

Body temperature and weight. As expected, body temperature ($F_{3,131}$=80.178, $P<0.001$) and weight ($F_{3,131}$=382.203, $P<0.001$) varied with age. In addition, body temperature differed between genotypes ($F_{2,131}=5.061$, $P=0.008$; genotype x development: $F_{6,131}$=1.230, $P=0.295$). Overall, *Shank1*$^{-/-}$ pups had lower body temperatures than *Shank1*$^{+/-}$ ($P=0.001$) and *Shank1*$^{+/+}$ controls ($P=0.018$), with the latter not differing ($P=0.291$). However, on individual PNDs no genotype differences were detected (all *P*-values >0.050; Fig. 5A). Also, body weight differed between genotypes ($F_{2,131}=11.699$, $P<0.001$; genotype × development: $F_{6,131}=1.983$, $P=0.072$). Overall, *Shank1*$^{-/-}$ pups had lower body weights than *Shank1*$^{+/-}$ ($P<0.001$) and

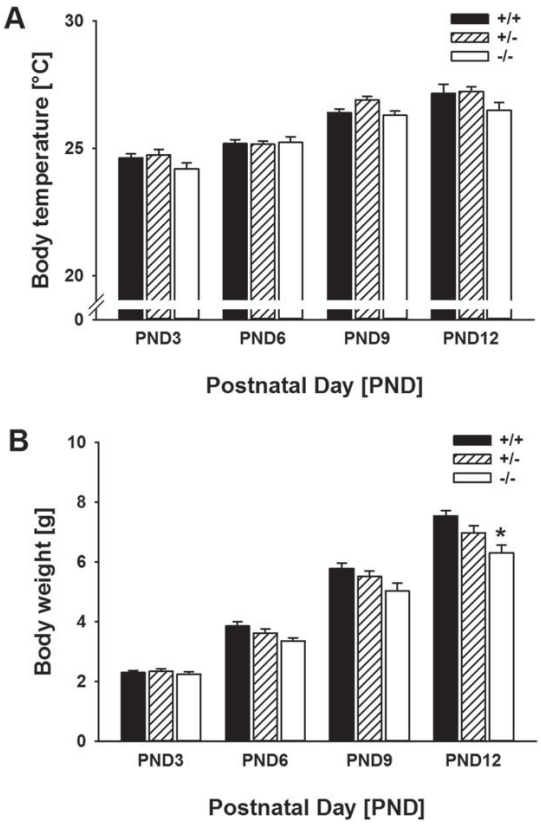

Figure 5. Experiment I: Body temperature and weight in isolated *Shank1* pups. (A) Body temperature (in Celsius degrees [°C]) and (B) body weight gain (in grams [g]) in pups tested for isolation-induced USV on individual postnatal days [PND], namely PND3, PND6, PND9, and PND12. Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Group sizes (for individual PNDs): *Shank1*$^{+/+}$ mice: $N_{PND3} = 12$, $N_{PND6} = 15$, $N_{PND9} = 14$, $N_{PND12} = 12$; *Shank1*$^{+/-}$ mice: $N_{PND3} = 13$, $N_{PND6} = 15$, $N_{PND9} = 13$, $N_{PND12} = 13$; *Shank1*$^{-/-}$ mice: $N_{PND3} = 14$, $N_{PND6} = 12$, $N_{PND9} = 11$, $N_{PND12} = 11$. Data are presented as means + standard errors of the mean. *$P < 0.050$ vs. *Shank1*$^{+/+}$.

Shank1$^{+/+}$ controls ($P < 0.001$), with *Shank1*$^{+/-}$ pups also having lower body weights than *Shank1*$^{+/+}$ controls ($P = 0.025$). Genotype differences in body weight emerged over time, with no differences on PND3, PND6, and PND9 (all *P*-values >0.050), but on PND12 ($F_{2,30} = 6.903$, $P = 0.003$). On PND12, *Shank1*$^{-/-}$ pups had lower body weights than *Shank1*$^{+/-}$ pups ($P = 0.001$) and tended to have lower body weights than *Shank1*$^{+/+}$ controls ($P = 0.050$), with the latter also tending to differ ($P = 0.089$; Fig. 5B). Finally, body temperature also differed between sexes ($F_{1,131} = 4.036$, $P = 0.047$; sex × development: $F_{3,131} = 0.291$, $P = 0.832$), with females constantly having higher body temperatures than males (genotype × sex: $F_{2,131} = 0.333$, $P = 0.718$; genotype × sex × development: $F_{6,131} = 0.753$, $P = 0.608$). Sex had no effect on body weight (all *P*-values >0.050; not shown). No evidence for genotype or sex effects was obtained for somatosensory reflexes (for details: Supporting Information).

Experiment II: Effects of Social Context

Clean bedding. As observed in Experiment I on PND9, where pups were also tested on clean bedding, numbers of isolation-induced USV emitted by pups did not differ between genotypes ($F_{2,30} = 0.456$, $P = 0.638$; Fig. 6A). During isolation, mouse pups slightly increased their emission rates over time irrespective of genotype (Fig. 6A′).

Home cage bedding. Under the home cage bedding condition, however, USV emission rates differed between genotypes ($F_{2,18} = 5.181$, $P = 0.017$). The numbers of USV emitted by *Shank1*$^{-/-}$ pups were reduced as compared to *Shank1*$^{+/-}$ ($P = 0.046$) and *Shank1*$^{+/+}$ controls ($P = 0.003$), whereas the latter did not differ ($P = 0.184$; Fig. 6B). Mouse pups again slightly increased their emission rates over time, with USV emission being inhibited especially during the first half of testing in *Shank1*$^{-/-}$ pups (Fig. 6B′).

Male cage bedding. When pups were tested for isolation-induced USV on bedding from a male cage, numbers of USV emitted were affected by genotype ($F_{2,27} = 3.759$, $P = 0.036$). *Shank1*$^{-/-}$ pups emitted fewer USV than *Shank1*$^{+/-}$ ($P = 0.044$) and *Shank1*$^{+/+}$ controls ($P = 0.014$; Fig. 6C). Mouse pups again slightly increased their emission rates over time, with USV emission being overall reduced in *Shank1*$^{-/-}$ pups (Fig. 6C′).

Sex effects. Sex had no effect on isolation-induced USV emission irrespective of social context (all *P*-values >0.050; genotype × sex: all *P*-values >0.050).

Discussion

SHANK gene family members are among the most promising candidate genes for ASD [Guilmatre et al., 2014; Leblond et al., 2014] and several mouse models were created in order to investigate the contribution of *SHANK* genes to ASD, each of which reflects symptoms observed in autistic individuals to a certain extent [Jiang & Ehlers, 2013; Yoo, Bakes, Bradley, Collingridge, & Kaang, 2014]. As communication deficits are one of the major criteria for diagnosing ASD [American Psychiatric Association, 2013], yet still little is known about communication deficits in *Shank* mouse models for ASD [Wöhr, 2014], we focused on developmental and social aspects of ultrasonic communication in the *Shank1* mouse model by comparing *Shank1*$^{-/-}$ null mutant,

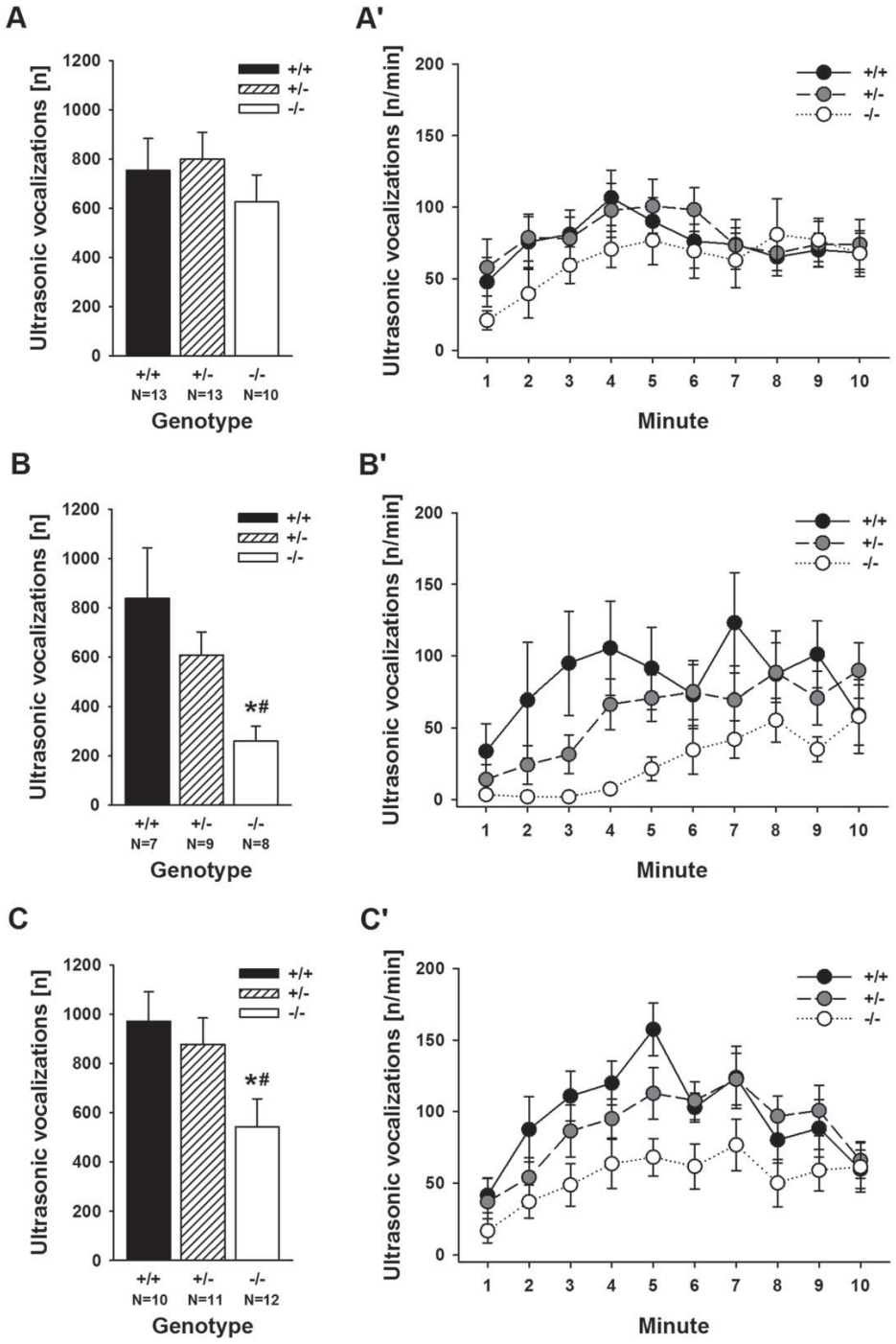

Figure 6. Experiment II: Effects of social odor context on ultrasonic vocalizations (USV) in isolated *Shank1* pups. Total number of USV [n] on postnatal day [PND] 9 under (A) clean bedding, (B) home cage bedding, and (C) male cage bedding conditions. Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Time course: Number of isolation-induced USV [n] in pups tested under (A′) clean, (B′) home cage bedding, and (C′) male cage bedding conditions per minute [min]. Black circles: *Shank1*$^{+/+}$ wildtype littermate control mice; grey circles: *Shank1*$^{+/-}$ heterozygous mice; white circles: *Shank1*$^{-/-}$ null mutant mice. Data are presented as means + standard errors of the mean. *$P < 0.050$ vs. *Shank1*$^{+/+}$; $^{\#}P < 0.050$ vs. *Shank1*$^{+/-}$.

Shank1$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate controls. The first experiment, where we evaluated the emission of isolation-induced USV on a developmental scale, revealed that *Shank1*$^{-/-}$ pups uttered fewer USV when isolated from mother and littermates, depicting a general communication deficit as previously reported [Wöhr et al., 2011]. Moreover, and in extension of Wöhr et al. [2011], who employed pups at a single stage of development (PND8), we compared genotypes across four different PNDs. Our analysis shows that the reduction in the number of USV emitted by *Shank1*$^{-/-}$ pups is further due to a delay in development. Call emission in *Shank1*$^{+/+}$ pups followed an inverted U-shaped pattern across test days, with number of USV peaking around PND6. While rate of calling appeared to have a similar pattern for USV emission in *Shank1*$^{-/-}$ pups, they displayed a shift with USV peaking around PND9. In turn, we observed genotype differences in call rate on PND6, where *Shank1*$^{-/-}$ pups emitted fewer USV than *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ littermates. Besides call rate, peak amplitude was among the other call parameters affected by genotype, with USV emitted by *Shank1*$^{-/-}$ pups being characterized by lower peak amplitudes than the ones emitted by *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ littermates, consistent with Wöhr et al. [2011]. Additional genotype differences were revealed by comparing *Shank1*$^{-/-}$ pups and *Shank1*$^{+/+}$ controls in a detailed subtype analysis through density plots, whereas the temporal organization of isolation-induced USV emission was not affected by genotype. Specifically, while in both genotypes one call cluster with isolation-induced USV characterized by peak frequencies between 50 and 80 kHz was identified on PND3, the segregation and formation of a new second cluster from PND6 on with peak frequencies between 80 and 100 kHz was more prominent in *Shank1*$^{-/-}$ pups. This is in line with the previous study focusing on PND8 only and showing that the reduced USV number observed in *Shank1*$^{-/-}$ pups on that day is mainly due to a reduction in the first call cluster [Wöhr, 2014], further supporting the notion that *Shank1* deletion affects communication on a developmental scale. Notably, observed genotype differences were independent from sex. While USV numbers differed between sexes in an age-dependent manner in Experiment I, our findings indicate an increased call rate in females only on PND9 and interactions between sex and genotype were not detected. Furthermore, no evidence for sex effects on USV emission were detected in Experiment II, with independent cohorts tested in three different social contexts. Together, our findings suggest that the effects of *Shank1* deletions on isolation-induced USV are not strongly affected by sex, with a slight tendency for more prominent genotype effects in females, as reported before [Wöhr et al., 2011].

The observed communication deficits in *Shank1*$^{-/-}$ pups are in line with alterations in ultrasonic communication in other *Shank* models. For instance, Schmeisser et al. [2012] analyzed USV emission between PND2 and PND12 and found that *Shank2*$^{-/-}$ females, but not males, called more than *Shank2*$^{+/+}$ females on PND4 and PND10. Similar to our results, in a follow-up study, Ey et al. [2013] reported that call rate was affected by genotype on PND6. Interestingly, the development of the call pattern very much resembles the picture in our study, with *Shank2*$^{-/-}$ mice displaying a shift in call rate peak. They also observed intact temporal organization in *Shank2*$^{-/-}$ mice [Ey et al., 2013]. Unlike *Shank1*$^{-/-}$ and *Shank2*$^{-/-}$ pups, however, no evidence for altered USV emission rates in mouse pups carrying a deletion of *Shank3* was obtained, with the typical inverted U-shaped developmental USV pattern being present in all genotypes [Yang et al., 2012]. It has to be emphasized, however, that the *Shank3* model tested in the study by Yang et al. [2012] displays only minor ASD-related behavioral phenotypes - in contrast to other *Shank3* models, in which isolation-induced USV were not assessed yet [Kouser et al., 2013, Peça et al., 2011; Wang et al., 2011].

One could argue that via deletion of *Shank1* an auditory deficit might be causing the *Shank1*$^{-/-}$ pups uttering fewer USV, since Braude et al. [2015] found evidence for expression of *Shank1*, but not *Shank2* and *Shank3*, in the cochlea. However, they did not detect any differences in auditory brainstem responses. Moreover, there is evidence that auditory input is not a prerequisite for the development of USV, as occurrence and structure of USV recorded from deaf otoferlin-knockout and hearing wildtype mice do not differ [Hammerschmidt et al., 2012]. One could further argue that genotype differences in body temperature might be causing the changes in USV emission. Overall, *Shank1*$^{-/-}$ pups had lower body temperatures than *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ controls, yet this was not detectable on individual PNDs. It therefore appears unlikely that the delay in the USV development observed in *Shank1*$^{-/-}$ pups could be due to alterations in body temperature. Furthermore, body weight was also affected by genotype. With *Shank1*$^{-/-}$ pups having lower body weights as compared to *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$, it is possible to attribute the reduced number of USV to decreased body size, as it has been speculated that body weight might play a role in USV features through increased thoracic size and/or lung capacity [Scattoni, Gandhy, Ricceri, & Crawley, 2008]. However, we could not detect any differences in body weight on PND6 where communication deficits were most prominent. Furthermore, on PND12, when genotype differences in body weight reached statistical significance the first time, no differences in isolation-induced USV were detected. Because of this double dissociation, it appears unlikely

that differences in USV emission are simply due to altered developmental profiles in body weight gain. In fact, the opposite might be true, as our developmental study shows that reduced emission rates of isolation-induced USV are followed but not preceded by slower body weight gain. It is well known that isolation-induced USV serve an important communicative function in regulating mother-offspring interactions, with such USV inducing maternal care behaviors, including search and retrieval behavior [Ehret & Haack, 1982; Sewell, 1970; Smith, 1976]. Moreover, it was shown that mothers are able to distinguish between different USV types and that they prefer certain types over others, indicating that acoustic parameters, such as call duration, peak amplitude, and peak frequency, affect the signal value of isolation-induced USV [Ehret & Haack 1982; Sewell, 1970; Smith, 1976; Wöhr et al., 2008]. Because *Shank1*$^{-/-}$ pups emit not just fewer isolation-induced USV, but also USV that are characterized by lower amplitudes it appears possible that *Shank1*$^{-/-}$ pups are less efficient in attracting mothers and inducing maternal care. Impaired ultrasonic communication in *Shank1*$^{-/-}$ pups might therefore explain slower body weight gain; a phenotype that emerges in the first two weeks of life when isolation-induced USV emission rates are typically highest. This might also explain to some extent the delay in motor development evident in *Shank1*$^{-/-}$ mice [Wöhr et al., 2011]. In this context, it has to be highlighted that heterozygous breeding pairs were used to obtain littermate controls and it is possible that subtle changes in maternal care behavior displayed by heterozygous mothers might have had an impact on the obtained result pattern. Currently, no detailed analysis of maternal care behavior in the *Shank1* mouse model for ASD is available.

Besides developmental factors, social odors play an important role in modulating pup USV emission [Branchi et al., 1998]. This is particularly relevant for mouse models for ASD, as social odor-induced modulation of pup USV could allow to assess ASD-relevant deficits in processing of social context information. In our second experiment, we focused therefore on the effects of social context on genotype differences in USV production by comparing clean bedding, home cage bedding, and male cage bedding. Our results show that, as in Experiment I, in the absence of any social component, *Shank1*$^{-/-}$ pups do not differ from their *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ littermates on PND9. Strikingly, however, communication deficits in *Shank1*$^{-/-}$ pups became evident when a social odor was present, regardless of the familiarity of this odor. *Shank1*$^{-/-}$ pups emitted almost no USV within the first minutes of exposure to the social context and started vocalizing later. These results are the first to demonstrate that the test environment plays a critical role in the production of isolation-induced USV in *Shank* mouse models for ASD. As communication by definition serves a social function, introducing social components could indeed bring about deficits in communication which might not be detectable in a nonsocial test environment. In future studies, it would therefore be interesting to assess isolation-induced USV in *Shank2*$^{-/-}$ and *Shank3*$^{-/-}$ mouse pups under social conditions.

One possible explanation for the more prominent genotype differences under social conditions could be that changing the social environment induces anxiety-related behavior, particularly the exposure to pheromones of an adult male. In fact, *Shank1*$^{+/+}$ littermates vocalized more when exposed to male cage bedding than during exposure to the other two contexts, which is in line with pharmacological, selective breeding, and behavioral studies linking anxiety-related behavior to increased isolation-induced USV [Kessler, Bosch, Bunck, Landgraf, & Neumann, 2011; Miczek, Weerts, Vivian, & Barros, 1995; Wöhr & Schwarting, 2008]. However, *Shank1*$^{-/-}$ pups displayed reduced and not increased levels of USV emission, as one might have expected based on earlier studies reporting higher anxiety levels in *Shank1*$^{-/-}$ mice [Hung et al., 2008; Silverman et al., 2011]. Another possible explanation is that changing the social environment affects the communication function of USV. Very early studies focusing on the effect of odors on pup USV hypothesized that cues associated with predators or a stranger male would inhibit ultrasonic calling as a possible strategy to avoid being detected [Conely & Bell, 1978; Lyons & Banks, 1982]. However, a later study by Elwood, Kennedy, and Blakely [1990] contradicted these findings and interpreted increased USV emission in response to male odor as a strategy for attracting the attention of the mother. In fact, remaining silent in the presence of a male odor might not necessarily increase the chances of survival, since in an environment where a pup can smell the potential danger it certainly can be vice versa [Santucci, Masterson, & Elwood, 1994]. In our study, *Shank1*$^{-/-}$ pups do not meet these criteria, as they remained rather silent in a potentially dangerous environment as compared to their *Shank1*$^{+/-}$ and *Shank1*$^{+/+}$ littermates. However, it is unclear why such deficits persist in a non-dangerous environment, namely home cage bedding. A simple interpretation would be that *Shank1*$^{-/-}$ pups are not able to differentiate the two odors and therefore remain silent regardless of the source of the odor. The ability of adult *Shank1*$^{-/-}$ mice to habituate and dishabituate to social odors, however, argues against this explanation [Silverman et al., 2011]. Of note, very little is known whether mouse models for ASD display deficits in their ability to identify nest odor from other social odors, such as a conspecific adult males [Moles et al., 2004].

While various species, including mice, developed a rather sophisticated acoustic communication system,

human language is profoundly different from the communication systems seen in other animals and constitutes a uniquely human trait. However, human language must have evolved, similarly to other complex abilities, through qualitative and quantitative modifications of morphological traits and neuronal networks already present in our ancestors and thus being the object of natural selection [Fitch, Huber, & Bugnyar, 2010; Scharff & Petri, 2011]. Although acoustic communication in mice is largely innate and auditory input is not required [Hammerschmidt et al., 2012, 2015; Wöhr et al., 2008; but see: Arriaga & Jarvis, 2013], which limits its suitability for studying genetic and neurobiological mechanisms underlying mental abilities required for human language and language deficits [Fischer & Hammerschmidt, 2011], mouse models may still provide novel insights through the identification of relevant ancestral mechanisms. Experimental evidence provided in the present study by means of the *Shank1* mouse model for ASD displaying alterations in the development of ultrasonic communication during early life might suggest that *SHANK1* is part of such an ancestral mechanism recruited and adapted to human language during evolution. In humans, *SHANK1* deletions were reported in males with a mild form of ASD with higher functioning not characterized by intellectual impairments and language deficits, at least when assessed at a later developmental stage with more than 5 years of age [Sato et al., 2012]. At an early developmental stage, however, impairments in language acquisition were seen in all males carrying a *SHANK1* deletion. Importantly, a role of *SHANK1* in language acquisition was further supported by a study showing that the T-allele of the *SHANK1* promotor variant rs3810280 is associated with reduced auditory working memory capacity in schizophrenia patients and subjects clinically at risk for developing a psychosis, but not healthy controls [Lennertz et al., 2012], consistent with the fact that *SHANK1* mutations were also linked to schizophrenia [Fromer et al., 2014]. Together, these results indicate that *SHANK1* is involved in acoustic communication across species, with genetic alterations in *SHANK1* resulting in social communication/interaction deficits.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to M.W. (DFG WO 1732/1-1). The authors wish to thank Jacqueline Crawley, University of California Davis School of Medicine, and the Howard Hughes Medical Institute investigators Albert Hung and Morgan Sheng for providing the *Shank1* mouse line. The authors also wish to thank Wiebke Dürichen, Clara Krzikalla, and Tobias Redecker for their help in this project.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Isolation-induced USV - Analysis: Peak frequency and peak amplitude were derived from the average spectrum of the entire call. Peak amplitude was defined as the point with the highest energy within the spectrum. Peak frequency was defined as the frequency at the location of the peak amplitude within the spectrum. The extent of frequency modulation was defined as the difference between the lowest and the highest peak frequency within each call. In addition, call subtypes were determined by means of density plots depicting peak frequency versus peak amplitude. Finally, to assess the temporal organization of isolation-induced USV emission, sequential analyses were performed by correlating the durations of given isolation-induced USV with the durations of the previous one (N-1), the ones two before (N-2), and the ones three before (N-3).

Somatosensory Reflexes: After the 10 min isolation period, surface righting and vertical screen holding were determined in pups from Experiment I. For surface righting, the pup was gently held on its back and released. Latency to flip over onto the abdomen with four paws touching the surface was measured with a stopwatch, with a maximum latency of 30 s. For vertical screen holding, the pup was placed on a square grid (8 x 11 cm) at 90° angle. Length of time the pup was able to stay on the grid was measured with a stopwatch, with a maximum latency of 30 s.

RESULTS

Experiment I: Developmental Aspects

Somatosensory Reflexes - Development: As expected, surface righting ($F_{3,131}$=61.279, p<0.001) and vertical screen holding ($F_{3,131}$=127.129, p<0.001) varied with age.

Somatosensory Reflexes - Genotype: Surface righting differed between genotypes in an age-dependent manner ($F_{2,131}$=0.380, p=0.684; genotype x development: $F_{6,131}$=2.232, p=0.044). When comparing genotypes on individual PNDs, no genotype differences were obtained on PND3, PND6, and PND12 ($F_{2,33}$=3.123, p=0.057; $F_{2,36}$=0.422, p=0.659 and $F_{2,30}$=1.277, p=0.293; respectively). However, a genotype effect was obtained on PND9 ($F_{2,32}$=4.777, p=0.015), yet individual comparisons between genotypes did not reach statistical significance (WT – HET: p=0.050, WT-KO: p=0.407; HET-KO: p=0.284). Finally, vertical screen holding was not affected by genotype ($F_{2,131}$=2.101, p=0.126; genotype x development: $F_{6,131}$=0.576, p=0.749).

Somatosensory Reflexes - Sex: Sex had no effect on surface righting and vertical screen holding (all p-values >0.100; genotype x sex: all p-values >0.100; genotype x sex x development: all p-values >0.100), with the exception of two trends for sex x development interactions (surface righting and vertical screen holding: $F_{3,131}$=2.220, p=0.089 and $F_{3,131}$=2.225, p=0.088, respectively).

Study III: Repetitive behaviors in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context

Journal of Neuroscience Methods 234 (2014) 92–100

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Clinical Neuroscience

Repetitive behaviors in the *Shank1* knockout mouse model for autism spectrum disorder: Developmental aspects and effects of social context

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HIGHLIGHTS

- Autism spectrum disorder (ASD) is characterized by repetitive patterns of behavior.
- Members of the *SHANK* gene family are promising candidate genes for ASD.
- Repetitive behavior in mouse models for ASD is typically assessed by self-grooming.
- The *Shank1* knockout mouse model for ASD displays changes in repetitive behavior.
- Most prominent genotype differences are detected in the social context.

ARTICLE INFO

Article history:
Received 17 December 2013
Received in revised form 22 April 2014
Accepted 1 May 2014
Available online 9 May 2014

Keywords:
Animal model
Neurodevelopmental disorders
Postsynaptic density
Repetitive behavior
Marble burying
Self-grooming

ABSTRACT

Background: Autism spectrum disorder (ASD) is characterized by persistent deficits in social behavior and communication, together with restricted and repetitive patterns of behavior. Several ASD candidate genes have been identified, including the *SHANK* gene family with its three family members *SHANK1*, *SHANK2*, and *SHANK3*.
Methods: Typically, repetitive behavior in mouse models for ASD is assessed by measuring self-grooming behavior. The first aim of the current study was to assess repetitive behaviors in *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice by means of a comprehensive approach, including the assessment of self-grooming, digging behavior, and marble burying. The second aim was to establish a test paradigm that allows for assessing the effects of social context on the occurrence of repetitive behaviors in a genotype-dependent manner. To this aim, repetitive behaviors were repeatedly tested on three consecutive days in distinct social contexts, namely in presence or absence of social odors.
Results: *Shank1*$^{+/-}$ heterozygous and to a lesser extent *Shank1*$^{-/-}$ null mutant mice displayed slightly elevated levels of self-grooming behavior as adults, but not as juveniles, with genotype differences being most prominent in the social context. In contrast to elevated self-grooming behavior, marble burying was strongly reduced in adult *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{-/-}$ null mutant mice across social contexts, as compared to adult *Shank1*$^{+/+}$ wildtype littermate controls.
Conclusion: The opposite effects of the *Shank1* deletion on the two types of repetitive behaviors are in line with a number of studies on repetitive behaviors in other genetic *Shank* models.

1. Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders that are characterized by persistent deficits in social behavior and communication across multiple contexts (American Psychiatric Association, 2013). In addition to social communication deficits, restricted and repetitive patterns of behavior, interests, or activities occur (American Psychiatric Association, 2013). Typically, they manifest as stereotyped or repetitive motor movements, use of objects, or speech, as well as insistence on sameness, inflexible adherence to routines, and ritualized patterns of behavior. Highly restricted and fixated interests, including unusual interests in sensory aspects of the environment, such as excessive smelling of objects, are also often prominent. Impairments

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http://dx.doi.org/10.1016/j.jneumeth.2014.05.003
0165-0270/

in adjusting behavior to suit different social contexts are common (American Psychiatric Association, 2013).

While tremendous progress has been made in recognizing and diagnosing ASD in recent years (Jones and Lord, 2013), the causes of ASD are still largely unknown. However, the high concordance rate between monozygotic twins (Folstein and Rutter, 1977; Posthuma and Polderman, 2013) supports a strong genetic component and several ASD candidate genes have been identified (Abrahams and Geschwind, 2008; State, 2010), including the *SHANK* gene family with its three family members *SHANK1*, *SHANK2* (*ProSAP1*), and *SHANK3* (*ProSAP2*) (Grabrucker et al., 2011; Guilmatre et al., 2014; Jiang and Ehlers, 2013; Ting et al., 2012; Yoo et al., 2013). Since Durand et al. (2007) first described mutations in *SHANK3* in patients with ASD, mutations in *SHANK1* (Sato et al., 2012), *SHANK2* (Berkel et al., 2010; Leblond et al., 2012; Pinto et al., 2010), and *SHANK3* (Boccuto et al., 2013; Dhar et al., 2010; Gauthier et al., 2009, 2010; Gong et al., 2012; Marshall et al., 2008; Moessner et al., 2007; Pinto et al., 2010; Schaaf et al., 2011; Waga et al., 2011) have been repeatedly reported in cases of ASD and schizophrenia patients with ASD traits. In addition, *SHANK3* maps to the 22q13.3 Phelan-McDermid deletion syndrome region (Wilson et al., 2003), a neurodevelopmental disorder typically characterized by deficits in language acquisition and other ASD features (Phelan, 2008). *SHANK* genes encode for a family of multidomain "master scaffolding proteins", which are localized in the postsynaptic density of excitatory glutamatergic synapses (Grabrucker et al., 2011; Kim and Sheng, 2004; Kreienkamp, 2008; Sheng and Kim, 2000). At the functional level, mutations in *SHANK* genes are therefore thought to ultimately translate into changes in the excitation/inhibition balance (Ebert and Greenberg, 2013; Toro et al., 2010).

As a consequence, *SHANK* gene family members are amongst the most important candidates for modeling ASD in rodents, with the main aim of gaining a better understanding of the roles of individual *SHANK* genes in the etiology of ASD by revealing the neurobiological mechanisms underlying ASD-relevant behavioral phenotypes, and thus paving the way for developing novel efficient treatments (Ecker et al., 2013; Murphy and Spooren, 2012). In fact, various genetic *Shank* models were generated within the last few years, including *Shank1*$^{-/-}$ (Hung et al., 2008), *Shank2*$^{-/-}$ (Schmeisser et al., 2012; Won et al., 2012), and *Shank3*$^{-/-}$ null mutant mice (Kouser et al., 2013; Peça et al., 2011; Schmeisser et al., 2012; Wang et al., 2011; Yang et al., 2012). Since the diagnostic criteria for ASD are defined purely behaviorally (American Psychiatric Association, 2013) and since reliable biomarkers have not been identified yet, the validity of genetic mouse models for ASD strongly depends on their behavioral phenotype. Therefore, in-depth longitudinal behavioral phenotyping is a key feature of the current translational research strategy; a strategy that requires sensitive behavioral test paradigms with high relevance to each category of diagnostic symptoms (Silverman et al., 2010). Within the last decade, a comprehensive set of behavioral assays for detecting deficits in mouse social and communication behavior across multiple contexts was developed, along with behavioral test paradigms that allow the reliable assessment of restrictive and repetitive patterns of behavior, interests, or activities (Bishop and Lahvis, 2011; Silverman et al., 2010; Wöhr and Scattoni, 2013). Typically, repetitive behavior in mouse models for ASD, as in genetic *Shank* models, is assessed by measuring self-grooming behavior (Drapeau et al., 2014; Kouser et al., 2013; Peça et al., 2011; Schmeisser et al., 2012; Silverman et al., 2011; Wang et al., 2011; Won et al., 2012; Yang et al., 2012), while repetitive digging and marble burying are comparatively rarely assessed (Kouser et al., 2013; Schmeisser et al., 2012; Won et al., 2012), although well-established paradigms exist (Thomas et al., 2009). Very little is known about the impact of the social context on repetitive behaviors since it is not systematically determined in the vast majority of studies, with only few exceptions (Moy et al., 2014; Ryan et al., 2010).

Up to now, four studies on the behavioral effects of a deletion on *Shank1* in mice were conducted (Hung et al., 2008; Silverman et al., 2011; Wöhr, 2014; Wöhr et al., 2011). Hung et al. (2008) observed a reduction in locomotor activity, deficient motor learning in the rotarod task, and elevated anxiety-related behavior in the open field and the light-dark box. They further reported impaired contextual fear learning, but normal cued fear learning (Hung et al., 2008). In a spatial learning task using a radial arm maze, Hung et al. (2008) found enhanced acquisition, but impaired retention of spatial memory. Wöhr et al. (2011) focused on communication deficits and found reduced isolation-induced ultrasonic calling in pups, often accompanied by a reduction in the temporal organization of call sequences, as revealed by a subsequent more detailed analysis (Wöhr, 2014). In adult mice, a lack of social modulation of male ultrasonic calling in response to female urine was obtained (Wöhr et al., 2011). Scent marking behavior, a measure of olfactory communication (Arakawa et al., 2008), was also reduced (Wöhr et al., 2011). Finally, Silverman et al. (2011) replicated the motor and anxiety phenotype reported by Hung et al. (2008) and reported normal social behaviors during reciprocal social interactions in juveniles and in the three chamber social approach task as adults, contrasting with the communication deficits observed by Wöhr et al. (2011). Olfactory information processing of social and non-social odors appeared to be intact and no evidence for excessive self-grooming behavior was obtained (Silverman et al., 2011). The latter is in contrast to most of the other genetic *Shank* models (for a detailed comparative overview on the behavioral phenotypes displayed by genetic *Shank* models see: Jiang and Ehlers, 2013; Yoo et al., 2013). However, it has to be noted that the self-grooming experiment by Silverman et al. (2011) is difficult to interpret as *Shank1*$^{+/+}$ wildtype littermate control mice engaged in comparatively high levels of self-grooming. Other measures of repetitive behavior were not determined and social context was not manipulated. Thus, the first aim of the current study was to assess repetitive behaviors in *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice by means of a comprehensive approach, including the assessment of self-grooming, digging behavior, and marble burying. The second aim was to establish a test paradigm that allows for assessing the effects of social context on the occurrence of repetitive behaviors in a genotype-dependent manner. To this aim, repetitive behaviors were repeatedly tested on three consecutive days in distinct social contexts, namely in presence or absence of social odors. Besides social context, developmental aspects were studied.

2. Materials and methods

2.1. Animals and housing

Repetitive behaviors in *Shank1*$^{-/-}$ null mutant mice with a targeted replacement of exons 14 and 15 encoding almost the entire PDZ domain were compared to *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{+/+}$ wildtype littermate control mice. Mice were obtained from mutant lines originally generated by Hung et al. (2008) on two independent background strains: C57BL/6J and 129SvJae. As high mortality rates were obtained in the C57BL/6J background strain and very low locomotion in the 129SvJae background strain (Hung et al., 2008; Silverman et al., 2011), the two lines were crossed for at least three generations to produce a mixed C57BL/6J × 129SvJae background for the *Shank1* mutation, consistent with the other studies focusing on this *Shank1* mutant (Hung et al., 2008; Silverman et al., 2011; Wöhr, 2014; Wöhr et al.,

2011). Using a heterozygous breeding protocol, *Shank1*$^{+/-}$ males and females were bred in a conventional vivarium at the Biomedical Research Center of the Philipps-University of Marburg, Germany. Approximately 2 weeks after pairing for breeding, females were individually housed and inspected daily for pregnancy and delivery. The day of birth was considered as postnatal day (PND) 0. After weaning on PND 21, mice were socially housed in groups of 2–6 with same-sex partners in polycarbonate Makrolon type III IVC cages (265 mm × 150 mm × 420 mm, 825 cm^2; Ehret, Emmendingen, Germany). Bedding and a wooden board were provided in each cage. Standard rodent chow and water were available ad libitum. The colony room was maintained on a 12:12 light/dark cycle with lights on at 06:00 h, at approximately 22 °C and 40–50% humidity. All mice used to assess repetitive behaviors were tested for isolation-induced ultrasonic vocalizations between PND 3 and 12 using a similar protocol as Wöhr et al. (2011). Pups were identified by paw tattoo, using non-toxic animal tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum Manufacturing Inc., Brockville, Canada). The ink was inserted subcutaneously through a 30 gauge hypodermic needle tip into the center of the paw. All procedures were conducted in strict compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the legal requirements of Germany. Procedures were approved by the ethical committee of the local government (Regierungspräsidium, Gießen, Germany).

2.2. Genotyping

Mouse tail snips were collected by dissecting ~0.3 cm of tail between PND 3 and 12. Tails were digested, genomic DNA was isolated and purified using the Qiagen DNAeasy Blood & Tissue Kit according to the manufacturer's instructions (Hilden, Germany). After the extraction, 2.0 μl of DNA in buffer containing ~250–400 μg of DNA was amplified by PCR using the GE Healthcare Illustra™ PuReTaq RTG PCR Bead Kit (Little Chalfont, UK). The following primers were used: CAA ACC CCC ATC GAG GAA TTC (wildtype forward), CCA GGA CTG ACT GGG CTA GC (wildtype reverse); GCT TGG GTG GAG AGG CTA TTC (neo forward); CAA GGT GAG ATG ACA GGA GAT C (neo reverse), with the wildtype primers recognizing sequences from the deleted segment and the neo primers amplifying a portion of the neo cassette introduced in the targeting vector. Denaturing, annealing, and extension steps were performed by means of a thermocycler (MyCycler, BioRad, Hercules, CA, USA), using the following protocol: 1 cycle at 95 °C for 120 s, 30 cycles of 95 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s, and 1 cycle of 72 °C for 300 s. For electrophoresis, the Sub-Cell GT system (BioRad, Hercules, CA, USA) and a 1.5% agarose gel stained with GelRed™ 3X (Biotium, Hayward, CA, USA) was used. The Gel Doc™ imaging system with a UV tray (BioRad, Hercules, CA, USA) was applied for automated gel documentation.

2.3. Repetitive behavior

Repetitive behaviors were assessed in two independent experiments. Both experiments were performed between 08:00 and 18:00 h under dim red light in a quiet behavioral test room. In Experiment I, juvenile *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice were tested on PND 42. In this experiment, each mouse was placed individually into a clean polycarbonate Makrolon type III IVC cage with a plastic top (265 mm × 150 mm × 420 mm, 825 cm^2) once for 15 min. The cage contained a layer of fresh bedding material. A digital camera (Sony Digital Camcorder 8; Minato, Japan) was placed approximately 60 cm in front of the cage with a slightly elevated view to record the sessions. A trained observer uninformed of the genotypes scored the videos with a stopwatch for the cumulative time spent grooming all body regions and the duration of digging behavior. Also, locomotor activity and rearing were quantified. For locomotor activity, the cage was divided into two virtual halves, and the numbers of line crosses between these two halves were counted. Rearing behavior was quantified as the number of times the subject mouse reared on its hind legs. In Experiment II, adult *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice were tested between PND 155 and 174. As in Experiment I, each mouse was placed individually into clean polycarbonate Makrolon type III IVC cage with a plastic top (265 mm × 150 mm × 420 mm, 825 cm^2). In Experiment II, however, 20 glass marbles (diameter: 15 mm) were added equidistant in a 4 × 5 arrangement on top of a 4.5 cm layer of bedding material, following the protocol developed by Thomas et al. (2009). Mice were tested for 30 min. A 30 min test duration was used because the numbers of marbles buried following a 20 min test duration was found to be relatively low in a pilot study using *Shank1*$^{+/+}$ wildtype mice (completely buried: 1.88 ± 1.20; completely and half buried: 7.50 ± 2.02). Clearly higher numbers of marbles buried were found following a 30 min exposure (completely buried: 6.00 ± 1.51; completely and half buried: 13.85 ± 1.55). Marbles were thoroughly cleaned with acetic acid solution (0.1%) between individual subject mice. Testing was performed on three consecutive days and the social context was manipulated by the presence or absence of social odors. On the first day, fresh bedding was used. On the second day, soiled bedding from an unfamiliar cage housing mice of the same sex as the subject mouse was added on top of the layer of fresh bedding. Finally, on the third day, fresh bedding was used again. To record the sessions, a top mounted digital camera (Sony Digital Camcorder 8; Minato, Japan) was placed approximately 60 cm above the cage. A trained observer uninformed of the genotypes scored the videos with the behavioral analysis software The Observer XT10 (Noldus Information Technology, Wageningen, The Netherlands) for the cumulative time spent grooming all body regions and the duration of digging behavior. The numbers of self-grooming events and their duration were determined as well. In addition, locomotor activity and rearing were quantified, as described above. Finally, the numbers of completely buried marbles and the numbers of completely and half buried marbles were counted immediately following testing.

2.4. Statistical analysis

In Experiment I, ANOVAs with the between-subject factor genotype were used to compare self-grooming, digging behavior, locomotor activity, and rearing behavior between juvenile *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice. In Experiment II, ANOVAs for Repeated Measurements with the between-subject factor genotype and the within-subject factor test day were applied to assess genotype differences between adult *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice across test days (first day: fresh bedding; second day: soiled bedding; third day: fresh bedding). When analyzing individual test days separately, ANOVAs with the between-subject factor genotype were used. For assessing changes across test days within individual genotypes, ANOVAs for Repeated Measurements with the within-subject factor test day were calculated. ANOVAs were followed by paired *t*-tests or LSD post hoc analysis when appropriate. Male and female data were combined, since sex differences were not observed (all *p*-values >0.050). A *p*-value of <0.050 was considered statistically significant. Corrected values were used in case Levene's test for equality of variances was significant.

3. Results

3.1. Experiment I

Juvenile *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice did not differ in self-grooming and digging behavior ($F_{2,34} = 1.804$; $p = 0.180$ and $F_{2,34} = 1.985$; $p = 0.153$; respectively; Fig. 1A and B), while locomotor activity, as assessed by means of line crossings, as well as rearing behavior differed significantly between genotypes ($F_{2,34} = 3.975$; $p = 0.028$ and $F_{2,34} = 12.470$; $p < 0.001$; respectively; Fig. 1C and D). *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice displayed fewer line crossings than *Shank1*$^{+/+}$ wildtype littermate controls ($p = 0.010$ and $p = 0.045$; respectively), with the former two not differing from each other ($p = 0.363$). Rearing behavior differed between genotypes in a dose-dependent manner. *Shank1*$^{-/-}$ null mutant mice reared significantly less often than *Shank1*$^{+/-}$ heterozygous mice ($p = 0.017$) and *Shank1*$^{+/+}$ wildtype littermate controls ($p < 0.001$), with the latter two also differing from each other ($p = 0.005$).

3.2. Experiment II

Adult *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice differed significantly in self-grooming and self-grooming changed across test days, indicating an effect of social context on repetitive behaviors (genotype: $F_{2,42} = 3.302$; $p = 0.047$; test day: $F_{2,84} = 9.657$; $p < 0.001$; genotype × test day interaction: $F_{4,84} = 2.131$; $p = 0.084$; Fig. 2A). Genotype differences in self-grooming were mainly due to an increase of the durations of individual self-grooming events (not shown), since the numbers of self-grooming events did not differ between genotypes (not shown). When analyzing individual test days separately, genotype differences were obtained for the first test day when mice were exposed to fresh bedding ($F_{2,42} = 4.054$; $p = 0.025$) as well as for the second test day when mice were exposed to soiled bedding ($F_{2,42} = 3.991$; $p = 0.026$), but not during the third test day when reexposed to fresh bedding ($F_{2,42} = 0.968$; $p = 0.388$). On the first and the second test day, *Shank1*$^{+/-}$ heterozygous mice displayed more self-grooming than *Shank1*$^{+/+}$ wildtype littermate controls ($p = 0.010$ and $p = 0.007$; respectively). *Shank1*$^{+/-}$ heterozygous mice also tended to display more self-grooming than *Shank1*$^{-/-}$ null mutant mice on the first test day during fresh bedding exposure ($p = 0.052$), with the latter being similar to *Shank1*$^{+/+}$ wildtype littermate controls ($p = 0.449$). When exposed to soiled bedding on the second test day, however, *Shank1*$^{-/-}$ null mutant mice displayed similar self-grooming levels as *Shank1*$^{+/-}$ heterozygous mice ($p = 0.305$) and tended to differ from *Shank1*$^{+/+}$ wildtype littermate controls ($p = 0.085$). The observed genotype differences are at least partly due to the fact that self-grooming in *Shank1*$^{+/+}$ wildtype littermate controls was affected by social context and differed between test days ($F_{2,24} = 13.699$; $p < 0.001$), which was not the case in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice ($F_{2,28} = 1.104$; $p = 0.345$ and $F_{2,32} = 1.078$; $p = 0.352$; respectively). In *Shank1*$^{+/+}$ wildtype littermate controls, self-grooming was lower on the second test day when mice were exposed to soiled bedding as compared to the first and third test day when fresh bedding was used ($t_{12} = 2.186$, $p = 0.049$ and $t_{12} = -5.291$, $p < 0.001$; respectively). Self-grooming was further found to be strongly elevated on the third test day in comparison with the first one ($t_{12} = -2.840$, $p = 0.015$). In contrast, digging behavior was not affected by genotype and did not differ between test days ($F_{2,42} = 0.685$; $p = 0.510$; test day: $F_{2,84} = 0.608$; $p = 0.547$; genotype × test day interaction: $F_{4,84} = 0.800$; $p = 0.529$; Fig. 2B).

The numbers of completely buried marbles differed significantly between genotypes and changed across test days, again indicating an effect of social context on repetitive behaviors (genotype: $F_{2,42} = 9.112$; $p = 0.001$; test day: $F_{2,84} = 1.989$; $p = 0.143$; genotype × test day interaction: $F_{4,84} = 2.733$; $p = 0.034$; Fig. 3A). When analyzing individual test days separately, genotype differences were obtained for all three test days (first test day: $F_{2,42} = 7.237$; $p = 0.002$; second test day: $F_{2,42} = 10.698$; $p < 0.001$; third test day: $F_{2,42} = 4.233$; $p = 0.021$). On all three test days and thus irrespective of social context, *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice buried fewer marbles than *Shank1*$^{+/+}$ wildtype littermate controls (first test day: $p = 0.001$ and $p = 0.003$; respectively; second test day: $p < 0.001$ and $p = 0.003$; respectively; third test day: $p = 0.020$ and $p = 0.010$; respectively). *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice did not differ from each other (first test day: $p = 0.662$; second test day: $p = 0.109$; third test day: $p = 0.835$). Very similar results were obtained using a less conservative method to quantify the numbers of marbles buried, namely when including not only completely but also half buried marbles (genotype: $F_{2,42} = 5.663$; $p = 0.007$; test day: $F_{2,84} = 4.388$; $p = 0.015$; genotype × test day interaction: $F_{4,84} = 1.425$; $p = 0.233$; Fig. 3B). Again, genotype differences were obtained on all three test days, irrespective of social context (first day: $F_{2,42} = 3.498$; $p = 0.039$; second day: $F_{2,42} = 6.123$; $p = 0.005$; third day: $F_{2,42} = 3.565$; $p = 0.037$; not shown in detail).

Besides measures of repetitive behaviors, locomotor activity, as assessed by means of line crossings, and rearing behavior were also determined. Locomotor activity differed significantly between genotypes and changed across test days, indicating an effect of social context on exploratory behavior (genotype: $F_{2,42} = 3.439$; $p = 0.041$; test day: $F_{2,84} = 28.748$; $p < 0.001$; genotype × test day interaction: $F_{4,84} = 1.673$; $p = 0.164$; Fig. 4A). When analyzing individual test days separately, genotype differences were obtained for the first test day ($F_{2,42} = 5.696$; $p = 0.006$), but not the second or third test day ($F_{2,42} = 1.814$; $p = 0.175$ and $F_{2,42} = 2.456$; $p = 0.098$; respectively). On the first test day, *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice displayed fewer line crossings than *Shank1*$^{+/+}$ wildtype littermate controls ($p = 0.002$ and $p = 0.014$; respectively), while not differing from each other ($p = 0.436$). Comparisons across test days further indicate an effect of social context on locomotor activity in *Shank1*$^{-/-}$ null mutant mice ($F_{2,28} = 12.631$; $p < 0.001$), *Shank1*$^{+/-}$ heterozygous mice ($F_{2,32} = 31.235$; $p < 0.001$), and *Shank1*$^{+/+}$ wildtype littermate controls ($F_{2,24} = 5.170$; $p = 0.014$). In *Shank1*$^{+/+}$ wildtype littermate controls, locomotor activity was equally high during the first test day when exposed to fresh bedding and during the second test day with soiled bedding ($t_{12} = -0.046$, $p = 0.964$), but markedly dropped during the third test day, again with fresh bedding, as compared to the first and second one ($t_{12} = 3.537$, $p = 0.004$ and $t_{12} = 2.753$, $p = 0.018$; respectively). A similar temporal pattern was obtained in *Shank1*$^{+/-}$ heterozygous mice, with no change from the first to the second test day ($t_{16} = -0.494$, $p = 0.494$), but a marked reduction in locomotor activity during the third test day, as compared to the first and second one ($t_{16} = 7.298$, $p < 0.001$ and $t_{16} = -7.256$, $p < 0.001$). However, a very different temporal pattern was obtained in *Shank1*$^{-/-}$ null mutant mice. Their locomotor activity increased from the first test day with fresh bedding to the second test day with soiled bedding ($t_{14} = -3.317$, $p = 0.005$). On the third test day, locomotor activity was found to be strongly decreased, with locomotor activity levels being clearly lower than on the second test day ($t_{14} = 4.393$, $p = 0.001$) and slightly lower than on the first test day ($p = 0.078$).

Similar to locomotor activity, rearing behavior differed significantly between genotypes and changed across test days, again indicating an effect of social context on exploratory behavior (genotype: $F_{2,42} = 6.829$; $p = 0.003$; test day: $F_{2,84} = 22.740$; $p < 0.001$; genotype × test day interaction: $F_{4,84} = 3.640$; $p = 0.009$; Fig. 4B). When analyzing individual test days separately, genotype differences were obtained for the first and third test day ($F_{2,42} = 8.749$;

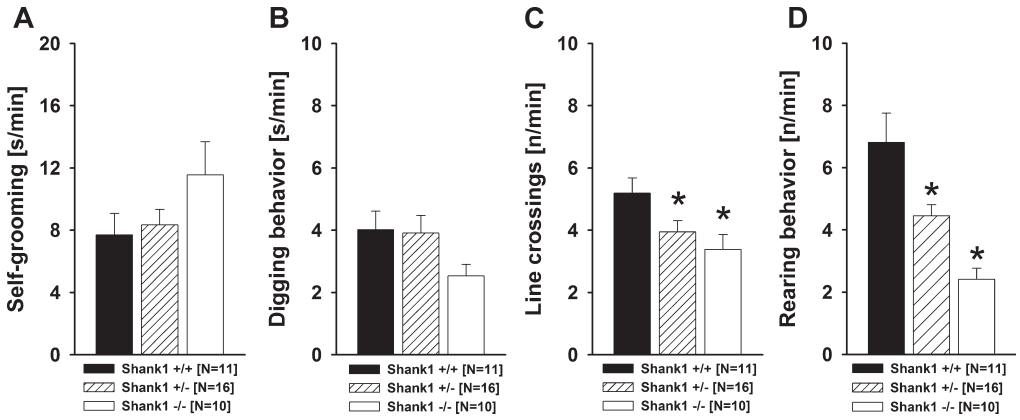

Fig. 1. Self-grooming (A), digging behavior (B), line crossings (C), and rearing behavior (D) in juvenile *Shank1* mice once exposed to fresh bedding. Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Data are presented as means ± standard errors of the mean. $^{*}p < 0.050$ vs. *Shank1*$^{+/+}$.

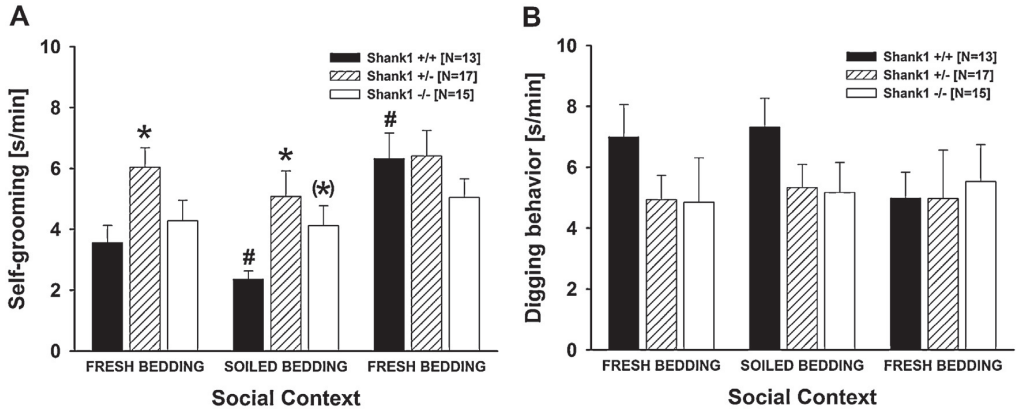

Fig. 2. Self-grooming (A) and digging behavior (B) in adult *Shank1* mice first exposed to fresh bedding (first test day, left), then soiled bedding (second test day, center), and finally fresh bedding again (third test day, right). Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Data are presented as means ± standard errors of the mean. $^{*}p < 0.050$ vs. *Shank1*$^{+/+}$; $^{\#}p < 0.050$ vs. first test day. Trends are shown in parentheses.

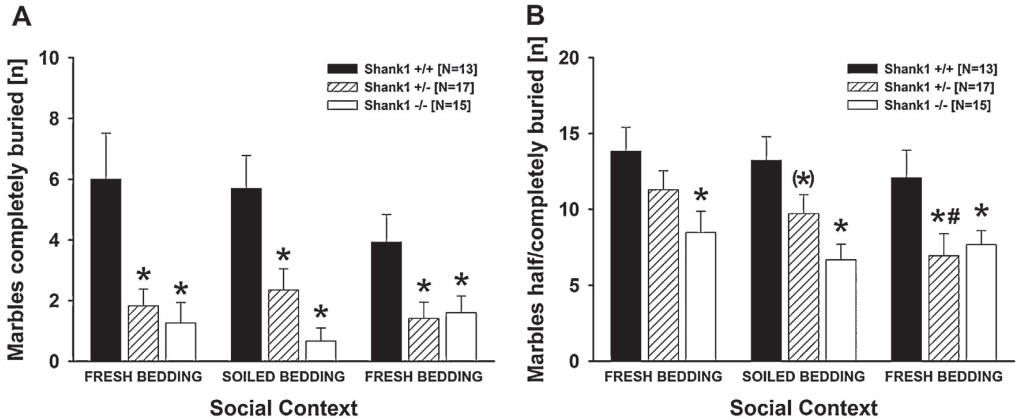

Fig. 3. Marbles completely buried (A) and marbles half or completely buried (B) in adult *Shank1* mice first exposed to fresh bedding (first test day, left), then soiled bedding (second test day, center), and finally fresh bedding again (third test day, right). Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Data are presented as means ± standard errors of the mean. $^{*}p < 0.050$ vs. *Shank1*$^{+/+}$; $^{\#}p < 0.050$ vs. first test day. Trends are shown in parentheses.

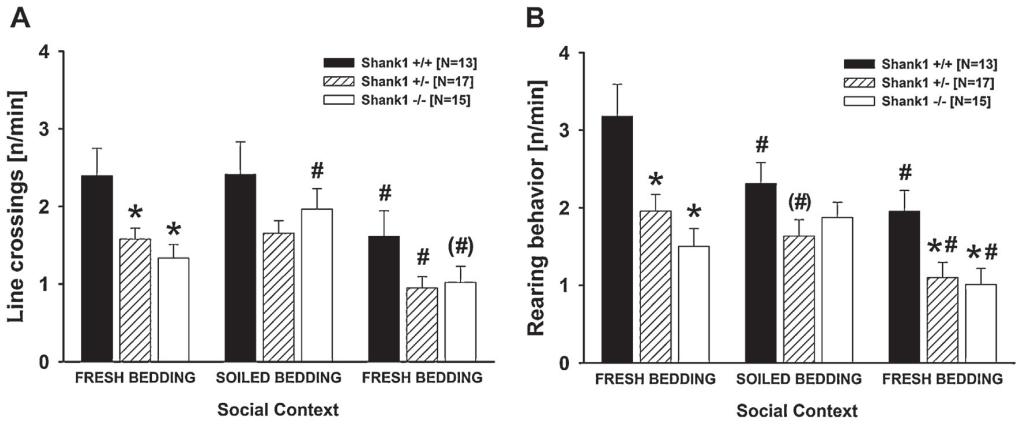

Fig. 4. Line crossings (A) and rearing behavior (B) in adult *Shank1* mice first exposed to fresh bedding (first test day, left), then soiled bedding (second test day, center), and finally fresh bedding again (third test day, right). Black bar: *Shank1*$^{+/+}$ wildtype littermate control mice; striped bar: *Shank1*$^{+/-}$ heterozygous mice; white bar: *Shank1*$^{-/-}$ null mutant mice. Data are presented as means ± standard errors of the mean. $^{*}p < 0.050$ vs. *Shank1*$^{+/+}$; $^{\#}p < 0.050$ vs. first test day. Trends are shown in parentheses.

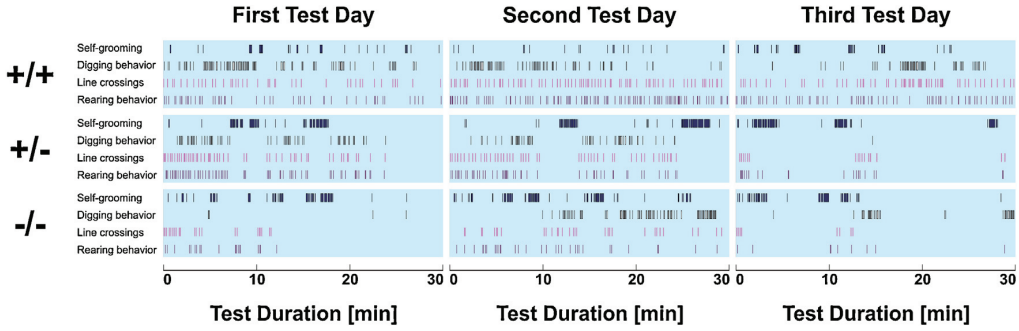

Fig. 5. Representative ethograms of adult *Shank1* mice first exposed to fresh bedding (first test day, left), then soiled bedding (second test day, center), and finally fresh bedding again (third test day, right). +/+: *Shank1*$^{+/+}$ wildtype littermate control mice; +/−: *Shank1*$^{+/-}$ heterozygous mice; −/−: *Shank1*$^{-/-}$ null mutant mice.

$p = 0.001$ and $F_{2,42} = 5.161$; $p = 0.010$; respectively), both with fresh bedding, but not on the second test day when soiled bedding was used ($F_{2,42} = 2.259$; $p = 0.117$). On both test days with fresh bedding, *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice displayed less rearing behavior than *Shank1*$^{+/+}$ wildtype littermate controls (first test day: $p < 0.001$ and $p = 0.004$; respectively; third test day: $p = 0.006$ and $p = 0.009$; respectively), while not differing from each other ($p = 0.244$ and $p = 0.436$; respectively). As for locomotor activity, comparisons across test days further indicate genotype-dependent effects of social context on rearing behavior, with prominent but distinct temporal patterns in *Shank1*$^{-/-}$ null mutant ($F_{2,28} = 9.025$; $p = 0.001$), *Shank1*$^{+/-}$ heterozygous ($F_{2,32} = 13.762$; $p < 0.001$), and *Shank1*$^{+/+}$ wildtype littermate control mice ($F_{2,24} = 8.014$; $p = 0.002$). In *Shank1*$^{+/+}$ wildtype littermate controls, rearing behavior decreased from the first to the second test day ($t_{12} = 2.566$, $p = 0.025$), remaining at about the same level during the third test day ($t_{12} = 1.562$, $p = 0.144$), still being lower as on the first test day ($t_{12} = 3.390$, $p = 0.005$). A similar, but less pronounced temporal pattern was obtained in *Shank1*$^{+/-}$ heterozygous mice, with a trend for a reduction from the first to the second test day ($t_{16} = 1.992$, $p = 0.073$). In contrast to *Shank1*$^{+/+}$ wildtype littermate controls, the most prominent decrease in *Shank1*$^{+/-}$ heterozygous mice occurred from the second to third test day ($t_{16} = 3.204$, $p = 0.006$), with levels of rearing behavior being clearly lower than on the first test day ($t_{16} = 5.348$, $p < 0.001$). Finally, a very different temporal pattern was seen in *Shank1*$^{-/-}$ null mutant mice. Their rearing behavior did not decrease from the first to the second test day ($t_{14} = -1.738$, $p = 0.104$). In fact, their rearing behavior even slightly increased, resulting in the highest rearing activity on the second test day when exposed to soiled bedding, as seen for locomotor activity. Then, from the second to the third test day, rearing behavior strongly decreased ($t_{14} = 4.723$, $p < 0.001$), with rearing activity levels being lower than on the first test day ($t_{14} = 2.302$, $p = 0.037$). Representative ethograms are shown in Fig. 5.

4. Discussion

While no evidence for genotype effects on repetitive behaviors was obtained in juvenile mice, adult *Shank1*$^{-/-}$ null mutant, *Shank1*$^{+/-}$ heterozygous, and *Shank1*$^{+/+}$ wildtype littermate control mice differed significantly in self-grooming behavior, with genotype differences being most prominent in the social context. Specifically, particularly high levels of self-grooming behavior were observed in *Shank1*$^{+/-}$ heterozygous mice, with self-grooming rates being elevated irrespective of social context on the first and second, but not third test day. In *Shank1*$^{-/-}$ null mutant mice, however, self-grooming behavior was comparatively high on the second test day when mice were exposed to soiled bedding only, with self-grooming levels being similar to the ones observed in *Shank1*$^{+/+}$ wildtype littermate controls on the first and third test day when fresh bedding was used. Together, this indicates that the detection of ASD-related genotype effects on self-grooming behavior might be facilitated when mice are tested in social contexts.

Elevated levels self-grooming behavior were mainly due to an increase of the durations of individual self-grooming events, since the numbers of self-grooming events did not differ between genotypes. The genotype effect on self-grooming behavior might be due to a reduction of GKAP/SAPAP in *Shank1*$^{-/-}$ null mutant mice, as compared to *Shank1*$^{+/+}$ wildtype littermate controls (Hung et al., 2008). GKAP/SAPAP interacts with PSD95 and Shank (Kreienkamp, 2008) and *Sapap3*$^{-/-}$ null mutant mice display extremely high levels of repetitive self-grooming behavior leading to facial hair loss and skin lesions (Welch et al., 2007). Moreover, variation within the human *SAPAP3* gene was found to be associated with obsessive–compulsive disorder and/or grooming disorders, including pathologic nail biting, pathologic skin picking, and/or trichotillomania (Bienvenu et al., 2009). In line with this, Sato et al. (2012) reported various repetitive behaviors, including hand flapping and stereotypic body movements, in male children with *SHANK1* deletions. However, it has to be highlighted that no data on GKAP/SAPAP levels in *Shank1*$^{+/-}$ heterozygous are available (Hung et al., 2008), in which the most prominent increases in self-grooming behavior were detected. The current findings are in contrast to the ones obtained by Silverman et al. (2011), who reported no change in self-grooming behavior in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice when comparing with *Shank1*$^{+/+}$ wildtype littermate controls and the mixed background strain C57BL/6J × 129SvJae. However, it has to be noted that the self-grooming experiment by Silverman et al. (2011) is difficult to interpret since *Shank1*$^{+/+}$ wildtype littermate controls and C57BL/6J × 129SvJae mice engaged in comparatively high levels of self-grooming. Overall, however, the genotype effects on self-grooming behavior are comparatively weak.

In contrast to self-grooming, digging behavior was not affected by genotype and the numbers of marbles buried were strongly reduced in adult *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice, as compared to *Shank1*$^{+/+}$ wildtype littermate controls. On all three test days, *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice buried clearly fewer marbles than *Shank1*$^{+/+}$ wildtype littermate controls. The opposite effects of the *Shank1* deletion on the two types of repetitive behaviors in mice appear to be unexpected, but are in line with a number of studies on repetitive behaviors in other genetic *Shank* models (Fig. 6). For instance, Schmeisser et al. (2012) reported increased self-grooming behavior in adult female *Shank2*$^{-/-}$ null mutant mice, while digging behavior was strongly decreased. In adult male *Shank2*$^{-/-}$ null mutant mice, self-grooming was unchanged, but digging behavior was again strongly decreased (Schmeisser et al., 2012). Similar findings were obtained by Won et al. (2012) in another *Shank2* model. While repetitive jumping behavior was increased in *Shank2*$^{-/-}$ null mutant mice of both sexes, digging behavior was strongly reduced, irrespective of sex. Self-grooming was not markedly affected by genotype (Won et al., 2012). Finally, in the only study in which both, self-grooming and digging behavior, were examined in a *Shank3* model, a similar pattern was reported. Self-grooming was elevated, at least in older mice, but marble burying was strongly reduced (Kouser et al., 2013). Together this shows that self-grooming and/or jumping behavior is unchanged or elevated in *Shank1*$^{-/-}$, *Shank2*$^{-/-}$, and *Shank3*$^{-/-}$ null mutant mice, while digging behavior and/or marble burying were consistently reduced in all model systems tested so far. This indicates that the reduced numbers of marbles buried are not simply due to the fact that *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice displayed a reduced level of locomotor activity, since some of the findings were obtained in *Shank* models with a similar reduction in locomotor activity (Kouser et al., 2013), but most the findings were obtained in *Shank* models characterized by hyperactivity (Schmeisser et al., 2012; Won et al., 2012). In fact, the numbers of marbles buried were found to be decreased in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice on all three test days in the current study, while genotype differences in locomotor activity were observed on the first test day only. The fact that the numbers of marbles buried differed between genotypes while digging behavior was similar in all three genotypes possibly indicates less efficient marble burying in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice than in *Shank1*$^{+/+}$ wildtype littermate controls.

In the current study, the observed genotype effects on repetitive behaviors appear to be more prominent in older mice. In juvenile mice, no evidence for genotype differences in self-grooming and digging behavior was obtained. This lack of genotype effects is probably not due to unexpected changes in the juvenile sample, since genotype effects on locomotor activity and rearing behavior were observed in the expected direction, consistent with previous studies in pups (Wöhr et al., 2011) and adult mice (Hung et al., 2008; Silverman et al., 2011; Wöhr et al., 2011). It is known that the level of self-grooming behavior changes throughout development. In C57BL/6J mice, self-grooming behavior peaks around 4–5 weeks of age, making it comparatively difficult to detect differences between mouse models for ASD, such as the inbred mouse strains BTBR T+tf/J and C58/J, and controls (McFarlane et al., 2008; Muehlmann et al., 2012; Ryan et al., 2010). In fact, Kouser et al. (2013) reported no genotype effect on self-grooming behavior in young adult *Shank3*$^{-/-}$ null mutant mice, but old mice with about one year of age, as compared to *Shank3*$^{+/+}$ wildtype littermate controls.

Besides developmental stage, test context affects the occurrence of repetitive behaviors. For instance, Thomas et al. (2009) showed that mice bury more marbles when tested in a novel cage with fresh bedding than in the home cage. Very little, however, is known about potential effects of the social context. Ryan et al. (2010; but see Muehlmann et al., 2012) reported abnormal high levels of repetitive behaviors in the C58/J mouse model for ASD, irrespective of whether they were tested in social or non-social test paradigms, yet Ryan et al. (2010) did not systematically manipulate this factor in their study. In a subsequent study, Moy et al. (2014) found that male C58/J mice display reduced marble burying behavior with rates being comparable in contexts with fresh bedding, with sweet cereals buried under the fresh bedding, and with a layer of soiled bedding from females under the fresh bedding. In the current study, self-grooming, but not digging behavior, changed across test days. The observed changes in self-grooming behavior were dependent on the presence or absence of social odors and not simply due to an overall habituation effect. In fact, a habituation effect characterized by a gradual decrease in self-grooming can be ruled out since levels of self-grooming behavior on the third test day were similar to those on the first test day or even higher. Hence, observed changes in self-grooming behavior across test days reflect the effect of social context. Importantly, this effect of social context on self-grooming behavior was found to be genotype-dependent. Most prominent genotype differences were detected in the social context on the second day, with *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice displaying comparatively high levels of self-grooming behavior. In *Shank1*$^{+/+}$ wildtype littermate controls, self-grooming behavior was lower on the second test day when mice were exposed to soiled bedding as compared to the first and third test day when fresh bedding was used. In contrast, levels of self-grooming behavior were unchanged and thus comparatively high in the social context in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice. This parallels recent findings by Wöhr et al. (2011) showing that the emission of ultrasonic vocalizations induced by female urine does not change in male *Shank1*$^{-/-}$ null mutant mice dependent on social factors, namely previous interactions with females. Previous female contact is well known in modulating the emission of ultrasonic vocalizations in adult male mice (Dizinno et al., 1978; Guo and Holy, 2007; Maggio et al., 1983;

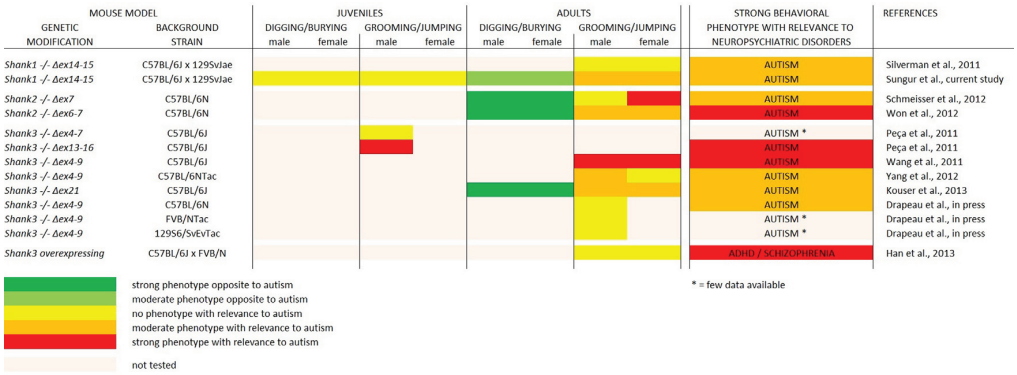

Fig. 6. Mosaic overview depicting findings obtained in studies on repetitive behaviors in *Shank* mouse models for autism spectrum disorder (ASD). Color coding reflects the strength of behavioral alterations with relevance to ASD. The summary score "strong phenotype with relevance to psychiatric disorders" was obtained as follows: For ASD, deficits in each of the three behavioral domains affected, social behavior, communication, and repetitive and stereotyped patterns of behavior, were rated as present (+1), absent (0), or opposite to expected ASD phenotype (−1), with a total score of >1 being a "strong phenotype with relevance to autism", a total score of >0 being a "moderate phenotype with relevance to autism", a total score of 0 being "no phenotype with relevance for autism", a total score of <0 being a "moderate phenotype opposite to autism", and a total score of <1 being a "strong phenotype opposite to autism". Values were averaged across sexes and studies (Han et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nyby et al., 1983; Roullet et al., 2011; Sipos et al., 1992, 1995), which serve a pro-social communicative function as social contact calls and attract female mice (Hammerschmidt et al., 2009; Pomerantz et al., 1983). Such impairments in adjusting behavior to suit different social contexts are common in ASD (American Psychiatric Association, 2013). While in healthy human subjects the occurrence of repetitive patterns of behavior is reduced in social contexts (Asendorpf, 1980), it was suggested that levels of repetitive behaviors are unchanged or even increased in children with ASD exposed to a social context (Baron-Cohen, 1989; Carruthers, 1996). It is believed that repetitive patterns of behavior help to reduce elevated anxiety levels that result from a primary deficit in the ability to understand social situations (Baron-Cohen, 1989; Carruthers, 1996). This view is supported by some autobiographical reports by individuals with ASD (for an overview on the autobiographical reports and the herewith often inconsistent empirical findings see: Turner, 1999). The present results are in line with this view.

While self-grooming behavior was clearly affected by social context in a genotype-dependent manner, the most prominent effect on marble burying was a main effect of genotype, with *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice burying fewer marbles than *Shank1*$^{+/+}$ wildtype littermate controls on all three test days. However, the overall lack of strong effects of social context on self-grooming and marble burying does not mean that *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice were not affected by social context. For instance, despite an often strongly reduced level of locomotor activity and rearing behavior in line with the literature (Hung et al., 2008; Silverman et al., 2011; Wöhr et al., 2011), *Shank1*$^{-/-}$ null mutant mice displayed a clear increase in locomotor activity and slightly elevated levels of rearing behavior on the second test day when exposed to soiled bedding, as compared to the first test day with fresh bedding. This is in contrast with the temporal pattern obtained in *Shank1*$^{+/+}$ wildtype littermate controls, in which locomotor activity and rearing behavior were unchanged or reduced across test days, in line with the expected habituation. The observed increase in locomotor activity and rearing behavior in *Shank1*$^{-/-}$ null mutant mice, with a similar, but less pronounced response pattern in *Shank1*$^{+/-}$ heterozygous mice, clearly shows that the lack of social modulation of self-grooming behavior in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice is not due to olfactory deficits. They were clearly able to detect the change in social context. Intact olfactory abilities in the social domain in *Shank1*$^{-/-}$ null mutant and *Shank1*$^{+/-}$ heterozygous mice were recently reported by Silverman et al. (2011).

In summary, *Shank1*$^{+/-}$ heterozygous and to a lesser extent *Shank1*$^{-/-}$ null mutant mice displayed slightly elevated levels of self-grooming behavior as adults, but not as juveniles, with genotype differences being most prominent in the social context. In contrast to elevated self-grooming behavior, marble burying was strongly reduced in adult *Shank1*$^{+/-}$ heterozygous and *Shank1*$^{-/-}$ null mutant mice across social contexts, as compared to adult *Shank1*$^{+/+}$ wildtype littermate controls. The opposite effects of the *Shank1* deletion on the two types of repetitive behaviors are in line with a number of studies on repetitive behaviors in other genetic *Shank* models.

Acknowledgements

This work was supported by a grant by the German Research Foundation (Deutsche Forschungsgemeinschaft) to M.W. (DFG WO 1732/1-1). The authors wish to thank Jacqueline Crawley, University of California Davis School of Medicine, and the Howard Hughes Medical Institute investigators Albert Hung and Morgan Sheng for providing the *Shank1* mouse line. The authors also wish to thank Clara Krzikalla and Max Rollwage for their help in data acquisition.

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6 APPENDIX

6.1 SHANK1 – Gene and Protein Nomenclature

SHANK1	Gene symbol in human
Shank1	Gene symbol in mouse
***Shank1*$^{+/+}$**	Mouse carrying a full copy of *Shank1* gene on both alleles (Wild-type)
***Shank1*$^{+/-}$**	Mouse with a deletion of *Shank1* gene on one allele (Heterozygous)
***Shank1*$^{-/-}$**	Mouse with a complete deletion of *Shank1* gene on both alleles (Knockout)
SHANK1	Protein symbol in human and mouse

6.2 Abbreviations

ADHD	Attention-deficit hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANK	Ankyrin repeats
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
Ca^{2+}	Calcium ion, with a positive charge of 2
CAM	Cell adhesion molecules
CaMK	Calcium-dependent kinase
CNV	Copy number variants
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
FXS	Fragile X syndrome
GABA	γ-aminobutyric acid
ID	Intellectual disability
K^{+}	Potassium ion, with a positive charge of 1
kb	Kilobase
kD	Kilodalton

MAPK	Ras-mitogen-activated protein kinase
MeCP2	Methyl CpG-binding protein 2
mGluR	Metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
Na^+	Sodium ion, with a positive charge of 1
NLGN	Neuroligin
NMDA	N-methyl-D-aspartate
NRXN	Neurexin
PDZ	Protein-protein interaction domain, named after: PSD-95, Dlg1, and ZO-1
PND	Postnatal day
PRO	Proline-rich domain
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein of 95 kilodalton molecular weight
PV	Parvalbumin
SAM	Sterile alpha motif domain
SH3	Src homology 3 domain
SHANK	Src homology 3 and ankyrin repeat domains
SNP/SNV	Single nucleotide polymorphism/single nucleotide variant
TSC	Tuberous sclerosis complex
USV	Ultrasonic vocalizations

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ACKNOWLEDGEMENTS

This work would not have been possible without the help, support, and contribution of many people. My special gratitude goes to…

Dr. Markus Wöhr, for giving me the opportunity of being a member of this excellent research group, and introducing the beautiful world of mouse language and behavior to me. He has been the trigger of my work in the most needed time, patiently guided and encouraged me with a friendly attitude and expert advice, and immensely contributed to my scientific grow-up.

Prof. Dr. Rainer Schwarting, an inspiring scientist and a kind "El Cheffe", for building this great scientific environment, and for his valuable advice throughout my PhD.

Prof. Dr. Sören Krach, for kindly accepting to be the second examiner of this thesis, and for his contribution to the evaluation of the work done.

Jakob Vörckel, Tobias Redecker, Magdalena Jochner, Elena Andres, Wiebke Dürichen, Clara Krzikalla and Max Rollwage for their input to the manuscripts, experiments and analyses.

Dr. Ayşe Kılıç, Dr. Hani Harb, and Prof. Dr. Holger Garn from Institute of Laboratory Medicine, and Pathobiochemistry-Molecular Diagnostics, for their guidance and help in the molecular analyses.

All members of AG Behavioral Neuroscience: Alex, Dominik, Henrike, Juan, Lea, Liana, Luan, Marco, Maria, Maxim, Moria, Natalie, Sebastian, Shona, and Theresa, for the fun times and fruitful discussions.

Long days of testing would not have been easy without the support of many great people I have in my life. Thank you…

My dearest friends and roommates in Marburg: Amina, Elisa, Ersin, Ezgi, Franzi, Gizem, Hannah, Karim, Lotta, and Sören; and my friends abroad: Deno, Emel, Fatma Zehra, Gülçin, Okan, Patrick, and Pelin, for bringing joy to my life. I also owe a big hearty thanks to Deniz Ashan and Emre, for providing me a second home in Magdeburg and taking care of me at the most stressful final phase of my PhD.

A sincere gratitude undoubtedly goes to Ayşe for giving me the creative kick whenever and for whatever reason I was stuck.

Philipp, not only for his help in the translation of the summary of this thesis, but also just for being an awesome friend.

Onur, for being as he is: doing the invaluable contribution to this thesis by always being there for me with his love and patience.

Last but not least, my dearest family for providing me the emotional support, and always cheering me up whenever I needed. İyi ki varsınız.

CURRICULUM VITAE

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ERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Dissertation:

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